AN ALGIVOROUS SEA SLUG AS A NOVEL SAMPLING TOOL AND ITS IMPLICATIONS FOR ALGAL DIVERSITY, HERBIVORE ECOLOGY, AND INVASIVE SPECIES TRACKING

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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This project is truly a testament of scientific curiosity. Kimberly Conklin first proposed this research after seeing *Plakobranchus* in Maunalua Bay, and because of how invaded this area is by *Avrainvillea lacerata* (*A. amadelpha sensu* Brostoff), she was curious whether these slugs could be eating the non-native alga. It is that curiosity that was the spark that started this dissertation research. Only with this initial question, and Kim's assistance, guidance, and support, was this research possible. Those that know Kim know that she is an amazing friend, mentor, and phycologist, and I am incredibly lucky to have had her with in my early years and as a graduate student.

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ABSTRACT

The Bryopsidales is a diverse order of green algae that includes important members of the marine environment as carbon and nutrient cyclers, ecological engineers, and substrate providers. Because of their unicellular, yet macroscopic construction, and often diminutive stature (some <2 cm at maturity), reliable morphological characters are rarely available for clear species delineation. These issues serve to confound their identification and even collection using traditional sampling methods. To address these challenges, the algivorous sacoglossan sea slug *Plakobranchus* cf. *ianthobapsus* Gould was used as a novel sampling tool for the detection and identification of bryopsidalean algae in the Hawaiian Islands. This sea slug feeds on siphonous green algae and sequesters their chloroplasts, effectively becoming photosynthetic itself. Using a sequestration preference study (Chp. 1), *P.* cf. *ianthobapsus* was found to preferentially sequester chloroplasts from diminutive siphonous species versus larger and more abundant species, most likely due to behavioral and/or physiological constraints. Molecular assessment using cloning (Chp. 1) and metabarcoding (Chp. 2) of their stolen chloroplasts, or "kleptoplasts," demonstrated that *P.* cf. *ianthobapsus* sequesters chloroplasts from up to 23 algal species, several of which are putative new species or records to the Hawaiian Islands. Furthermore, kleptoplast metabarcode data supported little community dissimilarity among sites across the Main Hawaiian Islands (MHI). In contrast, a coral-dominated site had a significantly dissimilar community assemblage compared to algal-dominated sites, suggesting that this diminutive algal community is cryptic and widespread within algal-dominated environments. Additionally, these data confirmed *Plakobranchus* as the first herbivore of the invasive green alga *Avrainvillea lacerata* (=*Avrainvillea amadelpha* sensu Brostoff) in Hawai‘i and expanded its known range in the archipelago. While this

research expanded our understanding of kleptoplast source diversity, it is difficult to discuss herbivore host selection limitations without a complete algal inventory.

To better understand host selection by *P. ianthobapsus* and a more comprehensive assessment of algal community diversity, metabarcoding of epilithic turf algae was conducted, allowing a direct comparison of recovered *P.* cf. *ianthobapsus* kleptoplasts (Chps. 1 &2) and algal species available in its environments. These data also provide a fine scale assessment of *Avrainvillea* distributions, as previous data (Chp. 2) suggested that *A. lacerata* exhibits a cryptic and diminutive morphology. Thus, these data provide a bridge between the kleptoplast diversity studies (Chps. 1 & 2) and invasive species phylogenetic studies (Chps. 4 & 5). The algal community metabarcode data suggested that *P.* cf. *ianthobapsus* only uses ~23% of the siphonous green algal species available to it, implying that the slug has more specific host selection than previously thought. Further, these results suggested that there is a clear taxonomic delineation in host selection with abundant representation of taxa from across the order Bryopsidales as well as the siphonous green algal order Dasycladales. *P.* cf. *ianthobapsus* nearly exclusively utilizes species from the bryopsidalean suborder Halimedineae, the exception being the bryopsidinean taxon *Codum edule*. These data also provide evidence of three new populations of *A. lacerata* on the west coast of Maui – the first documented spread of the alga apart from the islands of Kaua‘i and O‘ahu.

The siphonous green alga *Avrainvillea lacerata* exhibits high morphological plasticity in Hawai‘i, making its identification difficult. A phylogenetic reconstruction using two plastid loci and incorporating type specimen genetic data suggested that this species, which had previously been morphologically identified as *A. amadelpha* by Brostoff (1989), is in fact *A. lacerata*, a cosmopolitan species found throughout the Caribbean and Indo-Pacific. Further,

these results suggested that the alga identified as *A. amadelpha* in the Mediterranean is not in fact that taxon, although its true identity remains elusive based on assessment of specimens collected near Libya and Tunisia. Assessment of populations across O‘ahu’s south shore suggested that *A. lacerata* has likely been introduced to the Main Hawaiian Islands at least twice with genotypic heterogeneity correlated with the east and west sides of the island.

In addition to the morphological and molecular assessment of the Hawai‘i and Mediterranean invasive species of *Avrainvillea*, a second *Avrainvillea* species was discovered from two urbanized estuaries on the south shore of O‘ahu in 2014 and 2017, respectively. Morphological and molecular assessment of specimens representative of the two populations supported the alga’s identification as *A. erecta* (Chp. 4). The alga was recovered from 12-40 m depths and was growing among the seagrass *Halophila decipiens*, and the siphonous green algae *Halimeda kanaloana* and *Udotea* sp. Because of the populations’ locations near harbors and high boat traffic areas, it is possible that the alga was introduced via ballast water or anchor entanglement. It is also possible that the alga rafted from the west Pacific as tsunami debris following the 2011 Tohoku earthquake. Because of the invasive nature of *A. lacerata* in Hawai‘i, continued monitoring and management of *A. erecta* is essential to mitigating any negative effects of its introduction.

Exploring this unique slug-algal relationship from both organisms’ perspectives allows a more comprehensive understanding of the marine environments and communities of which they are members. This system has broad implications for algal biodiversity, exploration of community assemblages, herbivore ecology, and invasive species management.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

1.1 Siphonous Green Algal Diversity & Ecology

Siphonous green algae represent approximately 685 extant species in the orders Bryopsidales and Dasycladales (Chlorophyta, Ulvophyceae) today (Guiry & Guiry 2018). Both orders are acellular in vegetative construction (Kaplan and Hagemann 1991; Kaplan 2001) but differ based on the presence of septae in the Dasycladales, which effectively compartmentalize portions of the cell to establish multicellularity in the reproductive thalli (Hillis-Colinvaux 1984), and the multinucleate nature of the Bryopsidales (Graham *et al.* 2009). With biomineralization of many taxa in these orders, a fossil record is fairly robust. Based on a five locus, time-calibrated phylogeny, these orders diverged from each other and underwent massive diversification during the Proterozoic era approximately 571 million years ago (Verbruggen *et al.* 2009). While many species that comprise these orders are fairly large and conspicuous, many are diminutive in stature (<2 cm at maturity). In addition to and and perhaps because of their size, diminutive taxa are quite cryptic with few reliable morphological characters to distinguish them. As a result of these challenges, these diminutive species are minimally described and have a modest presence in the literature (e.g. Littler & Littler 1990; Verbruggen et al. 2009; Leliaert et al. 2014; Verbruggen et al. 2017).

The diversity of siphonous green algae is largely due to the evolution of unique traits, which allowed them to become significant and persistent members of the marine benthic communities. Some of these traits include an acellular thallus construction (Vroom et al. 2001, Vroom & Smith 2001, Vroom et al. 2003, Smith et al. 2004), rapid responses to changes in environmental conditions and nutrient availability (Williams 1984, Lobban & Harrison 1994, Chisholm et al. 1996; Smith et al. 2004; Malta et al. 2005), robust protective chemical profiles

provided by secondary metabolites (ZoBell & Allen 1935; Paul & Fenical 1986; Smyrniotopoulos *et al.* 2003, Puglisi *et al.* 2007; Birrell *et al*. 2008; Ritson-Williams *et al.* 2009; Morrow *et al*. 2011,) and/or endophytic bacteria (Hollants et al. 2011), highly successful vegetative reproduction via fragmentation (Hillis-Colinvaux 1965, Walters & Smith 1994; Vroom *et al.* 2003; Wright & Davis 2006), complex reproductive strategies (Vroom & Smith 2003), and heteroplasty (Graham *et al.* 2009). These traits increase their fitness by enabling them to grow and recover from damage quickly and effectively, survive in environments with limited nutrients and fluctuating conditions, escape predation and competition, and recruit, regenerate, and proliferate at astounding rates. The Bryopsidales in particular occupy a multitude of ecological niches inherent to their biology, physiology, biosynthetic chemistry, and phylogeny, resulting in particular competitiveness. As community members, siphonous green algae play essential roles in ecosystem stability. Rhizophytic and psammophytic genera such as *Halimeda* J.V. Lamouroux are able to affect sedimentation and as a result, are fundamental to creating a benthic habitat for other organisms, especially in seagrass beds (Den Hartog 1971; Zieman 1982). In addition to sedimentation, the presence of bryopsidalean algae in the benthic community contributes to nutrient cycling (Littler *et al.* 2005). In these environments, they are responsible for the majority of primary productivity (Payri 1988, Garrigue 1995) and sediment production (Wefer 1980).

However, many of these traits can also result in devastating impacts on marine communities. These traits that have allowed these taxa to diversify and thrive also contribute to their ability to invade new environments, become invasive, and resist eradication, as has been demonstrated by *Caulerpa taxifolia* (M. Vahl) C. Agardh in the Mediterranean (Meinesz

et al. 2001), *Codium fragile* ssp. *tomentosoides* (van Goor) P.C. Silva (=*Codium fragile* (Suringar) Hariot) around the globe (Provan *et al.* 2005), and the invasive species of *Avrainvillea* Decaisne in Hawai‘i (Brostoff 1989; Peyton 2009). Rapid regeneration of these species via fragmentation, (often) extensive holdfasts, and fast growth makes manual removal largely ineffective as an eradication strategy. Their life history and holocarpic mode of reproduction, which result in a highly reduced or even microscopic juveniles, further confound eradication. Additionally, bryopsidalean chemical defenses are highly successful at deterring native herbivores and allow them to compete with the flora and fauna in their new environments, which makes natural biocontrol non-viable. Lastly, their ability to change the benthos via sedimentation influence can create new soft sediment habitats on top of hard substrate (Littler & Littler 2005). This ability to overtake hard substrate is easily demonstrated by the invasive *Avrainvillea lacerata* (=*A. amadelpha sensu* Brostoff) J. Agardh in Hawai‘i.

1.2 Non-Native *Avrainvillea* Species in Hawai‘i and Elsewhere

Avrainvillea was first identified in the Main Hawaiian Islands (MHI) in the shallow marine environment off the western shore of O‘ahu in 1981 (Brostoff 1989). By 1985 the alga was documented in Maunalua Bay on O‘ahu’s southeastern shore (Brostoff 1989). Today, the alga persists in both the mesophotic zone and much of the shallow coastline of O‘ahu, as well as several shallow environments around the neighboring island of Kaua‘i (Smith et al. 2002, Spalding 2012, Wade & Sherwood 2018). Upon its discovery on Kaua‘i, Smith et al. (2002) noted that the alga was growing cryptically and diminutively, suggesting that the non-native alga may be difficult to detect in environments it has recently invaded. Brostoff (1989) identified the alga as *Avrainvillea amadelpha* using morphological characters but noted that many of the characteristics of the alga also resembled three other *Avrainvillea* species: *A.*

hollenbergii Trono, *A. lacerata* J. Agardh, and *A. riukiuensis* Yamada, suggesting high morphological plasticity of the alga.

Additionally, a second species, distinct from that previously recorded in the MHI, was recovered from two urbanized estuaries neighboring major harbors, first in 2015 from Honolulu Harbor and its entrance channel and again in 2017 near Keʻehi Harbor, both of which are in Māmala Bay on the south shore of Oʻahu (Wade et al. *in revision*). The alga was clearly distinct from the previously recorded *Avrainvillea* species because of its single flabellate blade per individual and long, conical holdfast, but similarly was found in both deep (i.e. >25 m) and shallow (i.e. 12-15 m) environments. Interestingly, both environments had individuals exhibiting varying morphology: smaller plants, perhaps juvenile, were composed of loose, spherical assemblages of siphons, while larger individuals had the characteristic fan-shaped blade. This differing morphology emphasized the importance of combining morphological and molecular assessment for species identification.

Lastly, an alga identified as *Avrainvillea amadelpha* was recently recorded in the Mediterranean Sea. In 2014, Dr. Ahmed Al Fituri contacted the Sherwood lab for assistance in the identification of the alga and shared both specimens and photographs from a site offshore of Tivoli, Libya, which demonstrated that the alga had become quite prevalent and potentially invasive in the Mediterranean. Recent morphological work demonstrated that an alga collected from the Kerkennah Archipelago in Tunisia (<100 km from the site sampled by Dr. Al Fituri) that is most likely the same species collected by Dr. Fituri morphologically matched the "*Avrainvillea amadelpha*" recorded in the MHI (Verlaque et al. 2017). However, Verlaque et al. (2017) used only morphological characters for identification as their molecular assessment was unsuccessful. Both the Hawaiʻi and Mediterranean specimens exhibit similar

gross morphological plasticity: varied blade shape and margin, varied stipe length and prominence, and holdfast variability in response to colonization of hard or fast substrate. Thus, the inclusion of molecular techniques would be invaluable for an informed identification of the alga.

1.3 Relationship Between Algivorous Sea Slug & Siphonous Algae

Avrainvillea's morphological plasticity and invasion potential for *Avrainvillea* demonstrate the need for not only a thorough morphological and molecular study of the genus to better identify these three species, but also the exploration of creative sampling to better detect the spread of these species, particularly in the Hawaiian Islands. One candidate for such creative sampling and species detection, particularly of diminutive taxa, is the algivorous sea slug *Plakobranchus* cf. *ianthobapsus* Gould (hereafter *Plakobranchus*). *Plakobranchus* represents a species complex comprised of at least 10 species (Krug et al. 2013) but is currently recognized monospecifically as *Plakobranchus ocellatus* van Hasselt. *Plakobranchus* is a member of the gastropod order Sacoglossa, perhaps best known for exhibiting kleptoplasty, the phenomenon of stealing and retaining chloroplasts from algal hosts (Kawaguti and Yamasu 1965). Kleptoplasty is exclusive to sacoglossans within Animalia but is also observed in ciliates and dinoflagellates (Pillet et al 2011, Schoener and McManus 2012). Sacoglossans are equipped with highly derived teeth and a radula that are used to pierce algal cell walls and suck up the cytoplasm; tooth shape and size are likely to be correlated with algal host selection (Jensen, 1983, 1993, 1997; Marín and Ros, 2004; Händeler et al., 2009; Christa et al., 2014). Once ingested, chloroplasts are phagocytotically packaged in host membranes by glandular microtubules and stored in the digestive gland (Hirose 2005). These kleptoplasts have been reported to maintain photosynthetic ability for up to 11 months (Evertsen et al. 2007), although this is likely an overestimation based on the

assumption that kleptoplasts degrade in a linear fashion (Vieira et al., 2009, Jesus et al. 2010, Wade & Sherwood 2017).

The majority of sacoglossans use siphonous algae as kleptoplast sources, primarily from the green algal order Bryopsidales or the xanthophyte *Vaucheria* A.P. de Candolle (Christa et al. 2014). *Plakobranchus*, in particular, exclusively incorporates chloroplasts from the Bryopsidales and the sister order Dasycladales (Dunlap 1975, Maeda et al. 2012, Christa et al. 2013.4b, Wade & Sherwood 2017). *Plakobranchus* is an ideal candidate as a sampler of siphonous green algae because of this specificity of host selection, but also because of its generalist use of species within this order (Christa et al. 2014a, Wade & Sherwood 2018).

1.4 Dissertation Organization

With the objective of harnessing *Plakobranchus* cf. *ianthobapsus* to explore siphonous green algal diversity in the MHI, and with an emphasis of early detection of "*Avrainvillea amadelpha*" in Hawai‘i, the chapters of this dissertation are as follows:

1) *Plakobranchus* kleptoplast diversity at Hunakai Beach: A pilot study: Using Sanger sequencing and amplicon cloning techniques, kleptoplast diversity of *Plakobranchus* cf. *ianthobapsus* was examined at Hunakai Beach, O‘ahu using two chloroplast regions. Based on the results of this study, a sequestration preference study was conducted to assess the algivore's host selection. Concurrently, a molecular assessment of *P.* cf. *ianthobapsus* in Hawai‘i was conducted to determine the population's identity in light of the diversity and framework provided by Krug et al. (2013) and the accepted monospecificity of the genus taxonomically accepted at that time. The results of this chapter were published in the following peer-reviewed article:

Wade, R.M., Sherwood, A.R. 2017. Molecular determination of kleptoplast origins from the sea slug *Plakobranchus ocellatus* (Sacoglossa, Gastropoda) reveals cryptic bryopsidalean (Chlorophyta) diversity in the Hawaiian Islands. *J. Phycol.* 53: 465-75.

(note: collaboration with P. Krug after the publication resulted in the reference of *Plakobranchus ocellatus* as *P.* cf. *ianthobapsus*, hence the use of both names in this dissertation).

2) *Plakobranchus* kleptoplast diversity across the MHI. Building on the baseline data provided by Chapter I, a deeper examination of kleptoplast diversity in Hawai‘i was undertaken via high throughput sequencing of kleptoplast amplicons from *Plakobranchus* collected from 10 sites across the Main Hawaiian Islands (MHI) during two seasons. Additionally, a molecular assessment of the individuals collected during one of the seasons were barcoded to assess monospecificity of *Plakobranchus* in Hawai‘i and to determine whether significant differences in kleptoplast diversity correspond to differences in algivore identity, and therefore to conspecific sequestration preferences. The data produced by this study were also used to infer siphonous algal community dissimilarity across sites and to detect *Avrainvillea* in newly recorded locations. The results of this chapter were published in the following peer-reviewed article:

Wade, R.M., Sherwood, A.R. 2018. Updating *Plakobranchus* cf. *ianthobapsus* (Gastropoda, Sacoglossa) host use: Diverse algal-animal interactions revealed by NGS with implications for invasive species management. *Mol.Phylogen. Evol.* 128:172-81 doi: 10.1016/j.ympev.2018.07.010.

3) Algal community diversity and its implications. This chapter stems from the results of Chapters I and II and uses high throughput sequencing of two chloroplast amplicons derived from turf algal communities collected from 20 sites across the MHI during two seasons. The resulting data were used to 1) assess community assemblage and dissimilarity across sites, 2) determine the limits of *Plakobranchus* cf. *ianthobapsus* host use in Hawai‘i by comparing the community diversity with those recovered in Chapters I and II, and 3) to use the fine scale community sampling to further examine the potential spread of *Avrainvillea lacerata.*

4) Molecular systematics of *Avrainvillea* and invasive species identifications. This chapter of the dissertation examines the molecular systematics of *Avrainvillea* as a genus in order to 1) reconstruct a molecular phylogeny and determine phylogenetic species for the first time, particularly in reference to the morphometric analyses and resulting morphotype groups described by Olsen-Stojkovich (1985), and 2) provide molecular and morphological identifications for three non-native species of *Avrainvillea*: *“A. amadelpha”* and a species more recently discovered in 2015 in Hawai‘i, and the species identified as *A. amadelpha* in the Mediterranean.

5) *Avrainvillea* cf. *erecta* in urbanized estuaries on the south shore of O‘ahu. Identification of the more recently introduced species with brief ecological details are discussed in the following peer-reviewed article:

Wade, R.M., Spalding, H.L, Peyton, K.A., Foster, K., Sauvage, T. S., Ross, M., Sherwood, A. 2018. R. A new record of *Avrainvillea erecta* (Berkeley) A. Gepp & E. S. Gepp (Bryopsidales, Chlorophyta) in urbanized estuaries in the Hawaiian Islands. *Biodiversity Data Journal.* doi: 10.3897/BDJ.6.e21617.

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CHAPTER 2. Molecular determination of kleptoplast origins from the sea slug *Plakobranchus ocellatus* (Sacoglossa, Gastropoda) reveals cryptic bryopsidalean (Chlorophyta) diversity in the Hawaiian Islands

Rachael M. Wade and Alison R. Sherwood

2.1 Abstract

The sacoglossan sea slug species complex *Plakobranchus ocellatus* is a common algivore throughout the tropical Pacific, including the Hawaiian Islands. *Plakobranchus ocellatus* is kleptoplastic - it sequesters and retains algal chloroplasts - a characteristic that can be exploited to molecularly characterize diminutive bryopsidalean algae that are typically difficult to locate, collect, and identify. Previous DNA barcode analyses of both *P. ocellatus* and its kleptoplasts have been conducted primarily in the western Pacific and have only minimally sampled the most eastern populations in the Hawaiian Islands. Using two chloroplast markers *rbc*L and *tuf*A, kleptoplast samples from an O‘ahu population of *P. ocellatus* were amplified and cloned to identify their algal sources. *Plakobranchus ocellatus* sequester chloroplasts from up to 11 bryopsidalean algal species, all but one being diminutive in thallus size. Notably, eight of the detected algal species were new records to the Hawaiian Islands. A sequestration preference study demonstrated that the O‘ahu population of *P. ocellatus* preferentially sequesters chloroplasts from diminutive, epilithic taxa. Using *cox*I barcoding of *P. ocellatus* we showed the O‘ahu population to be part of a clade that includes sequences from the neighboring island Maui, Australia, and the Philippines. The use of *P. ocellatus* as a novel sampling tool allows the exploration of the green algal community diversity and composition at a fine scale.

Key index words: Bryopsidales, Hawai‘i, kleptoplasty, *Plakobranchus ocellatus*, *rbc*L, *tuf*A, Ulvophyceae

Abbreviations: BI, Bayesian inference; bp, base-pair; ML, maximum likelihood; *rbc*L, large subunit ribulose bis-phosphate carboxylase/oxygenase; *tuf*A, elongation factor *tuf*A.

2.2 Introduction

The Bryopsidales is a diverse order of green algae and includes species that are important components of the marine ecosystem as ecological engineers, sediment producers, carbon and nutrient cyclers, and substrate providers (Williams and Fisher 1985, Littler et al. 1988, Littler and Littler 1990, Vroom and Smith 2001, 2003, Littler et al. 2004). Although bryopsidalean algae have variable and unique sexual reproduction strategies (i.e. sporic and gametic meiosis, haplobioncy, and holocarpic reproduction), many genera are known for their fragmentation ability, as demonstrated in the successful and persistent invasions of *Caulerpa taxifolia* (Vahl) C.Agardh and *Codium fragile* subsp. *fragile* (Suringar) Hariot (Smith and Walters 1999, Vroom and Smith 2001, 2003, Provan et al. 2005, Graham et al. 2009). While the large, conspicuous genera of the Bryopsidales are common subjects of current phycological systematic studies (e.g. Boisset and Gallego 2015, do Nascimentos Santos and de Castro Nunes 2015, Kojima et al. 2015, Lee and Kim 2015), the more diminutive genera (<2 cm at maturity) (e.g. *Poropsis*, *Pseudochlorodesmis*, *Rhipidosiphon*) are often overlooked due to their small size. As a result, they are challenging to collect and identify using traditional methods. Furthermore, molecular and morphological studies that have included diminutive representatives have shown that some of these taxa harbor substantial cryptic diversity (Kooistra 2002, Verbruggen et al. 2009).

Sacoglossan sea slugs are primarily algivores and are widely distributed; populations persist in temperate, northern latitude locales such as Norway and Russia, but species diversity is highest in the tropical Indo-Pacific (Jensen 1997). Sacoglossans, also known as "sap sucking sea slugs," are known for kleptoplasty, or sequestration and retention of chloroplasts from algal hosts (Clark 1992). Kleptoplasty has also been observed in ciliates and dinoflagellates (Pillet et al. 2011, Schoener and McManus 2012), but among metazoans

is only known to occur in the Sacoglossa (Clark 1992). Sacoglossans use a powerful, specialized radula to pierce algal cell walls and suck out the cytoplasm. Chloroplasts are then phagocytized by microvilli and enveloped by cell membranes (phagosomes) to be stored in the digestive gland (Clark 1992, Williams and Walker 1999, Hirose 2005). Kleptoplasts can maintain functionality within a slug's tissues on time scales of hours to over a year (Rumpho et al. 2000, 2011, Evertsen et al. 2007, Händeler et al. 2009). Some research suggests kleptoplasty benefits the animal host by directly transferring metabolites and lipids from functional kleptoplasts to the animal, ultimately increasing their fitness and survivability (Williams and Walker 1999, Händeler et al. 2009, Yamamoto et al. 2013, Cruz et al. 2013, Akimoto et al. 2014, Pelletreau et al. 2014, Baumgartner et al. 2015, Pierce et al. 2015), but the extent of the plastid enslavement is debated (Christa et al. 2014a, b). These sea slugs feed on and sequester chloroplasts from mostly ulvophycean green algae, particularly from the orders Bryopsidales and Dasycladales (Händeler et al. 2009). Taxa in the green algal order Cladophorales have also been observed as host species for sacoglossans (Ansell et al. 1999, Williams and Walker 1999, Grzymbowski et al. 2007, Wägele et al. 2010). However, their inability to be detected in studies using molecular assessments of kleptoplast origin has prevented their confirmation as both food and kleptoplast sources.

The sea slug species complex *Plakobranchus ocellatus* van Hasselt is a kleptoplastic sacoglossan of interest for several reasons. First, a systematics study has found that the monotypic genus *Plakobranchus* should be revised to include description of up to 10 species (Krug et al. 2013). This intrageneric diversity indicates that there is potential for algal host specialization amongst species. Second, the species complex is widespread throughout the tropical Pacific, from the Philippines to as far east as the Hawaiian Islands. Third, individuals

are known to sequester chloroplasts from as many as eight different algal taxa with an average of 3-4 taxa per individual (Wägele et al. 2011, Maeda et al. 2012, Christa et al. 2013, 2014c). Previous DNA barcoding attempts for *P. ocellatus* included very limited Hawaiian representation (only two specimens from the island Maui, Krug et al. 2013). As the most eastern and isolated part of *P. ocellatus*' distribution, the Hawaiian Islands provide an opportunity to generate new information for both this species and its algal kleptoplast sources. Specifically, in this study we use *P. ocellatus* as a novel sampling tool to reveal newly reported diminutive bryopsidalean diversity from the Hawaiian Islands and to examine the phylogeography of *P. ocellatus* within a broader context of available *cox*I sequences.

2.3 Materials and methods

2.3.1 Specimen collection. *Plakobranchus ocellatus* specimens were collected during spring (23 March 2013, n=10), summer (03 July 2013, n=20), and fall (21 November 2013, n=39) from shallow, sandy areas within 20m of shore from Hunakai Beach, O'ahu, Hawai'i (21.262888°N, 157.783768°W). Specimens from all seasons were used for kleptoplast diversity assessment and fall 2013 specimens were also used in sequestration preference trials. Green algae were sampled from the collection site each time. Kleptoplasts were sampled immediately after collection from each slug using a novel, non-lethal technique: ethanol was slowly dripped into watchglasses containing *Plakobranchus* individuals to relax the slug's parapodia; lamellae were then lightly disturbed using fine forceps to extract kleptoplasts with minimal tissue damage to the slug. Kleptoplasts were sampled from five randomized locations (of approximately 1 mm^2) across the dorsal surface of each slug to account for the potential non-uniform kleptoplast distribution and diversity through the tissue. A small piece of slug posterior parapodium was also sampled for DNA barcoding. After sampling, slugs were monitored for 2-6 weeks in a 10- or 20-gallon, filtered aquarium at 22-25°C with artificial

32-ppt seawater (Instant Ocean® or PETCO® Premium Marine Salt Mix) and a grow light with dual 14-watt 6500K bulbs (30 μmol/m^2/s). No mortalities occurred during this monitoring period. This gentle, non-lethal sampling technique allowed slugs to recover and be used in the subsequent sequestration preference trial.

2.3.2 DNA extraction and amplification. Kleptoplast, slug, and algal DNA were extracted using a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). *Plakobranchus ocellatus* extracts were amplified for the metazoan cytochrome oxidase I (*cox*I) barcode (658 bp) using the sacoglossan *cox*I primers coxFS and coxRS (Christa et al. 2013, Table 2.1). Kleptoplast and green algal extracts were amplified for portions of the *tuf*A (protein synthesis elongation factor *Tu*, 714 bp) and 5' end of the *rbc*L (large subunit of the ribulose bisphosphate carboxylase/oxygenase gene, 565 bp) chloroplast genes. The *tuf*A marker is commonly used for green algal DNA barcoding and species delineation (Saunders and Kucera 2010, Leliaert et al. 2014), and was amplified using the primers tufA_alg_up and tufA_alg_do that were designed specifically for kleptoplast DNA amplification (Händeler et al. 2010, Table 2.1). The *rbc*L marker was amplified with the primers rbcLF and rbcLR (Pierce et al. 2006) using a modification of the touchdown protocol described therein (Table 2.1). Amplification success was determined by gel electrophoresis. Samples were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and were sequenced on an ABI 3730xl DNA Analyzer at either the Advanced Studies in Genomics, Proteomics and Bioinformatics Genomics Laboratory or the Biotech Core at the Pacific Biosciences Research Center of the University of Hawai'i at Mānoa.

2.3.3 *P. ocellatus* kleptoplast diversity. A subsample of five slugs was analyzed from each set of samples for assessment of kleptoplast diversity. Successful PCR products from each of the five slugs were cloned using Invitrogen TOPO TA Cloning kits with TOP10 chemically competent *E. coli* cells (Life Technologies, Carlsbad, CA, USA). Twelve clones from each slug kleptoplast PCR product were directly amplified and sequenced for both plastid markers.

2.3.4 *P. ocellatus* sequestration preference trial. Slugs collected during the fall season were starved to allow kleptoplast breakdown before being placed into the sequestration preference trial. Light was increased periodically during the starvation period by partially and later completely surrounding the aquarium with aluminum foil to encourage kleptoplast breakdown; irradiance was increased from 30 μmol/m^2/s to 50 μmol/m^2/s on day 10 and to 90 μmol/m^2/s on day 32. Irradiance levels were measured using a calibrated spherical (4π) quantum sensor (Underwater LI-193SA, LI-COR, Lincoln, NE, USA). Photosynthetic activity of kleptoplasts was measured weekly via photosystem II (PSII) maximum quantum efficiency, or yield (F_V/F_M), using pulse-amplified modulated fluorometry (junior-PAM by Heinz Walz GmbH, Effeltrich, Germany) (Händeler et al. 2010, Jesus et al. 2010, Cruz et al. 2013). Slugs were dark acclimated for 30 min at room temperature (25°C) before each slug's kleptoplast photosynthetic yield was measured in the dark at three different locations along the lamellae: directly behind the cardiac swelling, medially, and posteriorly. Mean values were graphed to visualize kleptoplast degradation and to predict and identify the point of negligible photosynthetic activity, which was defined as the point where a yield value of ≤0.100 was obtained (Jesus et al. 2010, Fig. 2.1). For the purpose of this study, it was assumed that loss of photosynthetic activity was coupled with both the absence of chlorophyll pigmentation in the slug tissue and the absence of plastid genetic signal. Additionally, based on the conclusion

of Maeda et al. (2012) that older kleptoplasts are stochastically replaced with newly sequestered ones, we assumed that any remnant kleptoplast material would be replaced or at the very least overwhelmed by the signal from newly sequestered kleptoplasts during the sequestration preference trial. After 76 days of starvation, 20 slugs reached negligible photosynthetic yield values (Fig. 2.1) and were placed into a sequestration preference trial at the Anuenue Fisheries Research Center at Sand Island, O‘ahu, and the remaining slugs were released back to their collection site. For the sequestration trial, each slug was placed in an individual aquarium; the aquaria were arranged in a complete randomized design in a large tank with flowing seawater to maintain water temperature. Each aquarium was equipped with its own filtered seawater source and air emitter and represented a replicate in one of the following treatments: psammophytic algae (aquarium with sterile sand and one plant each of *Avrainvillea* sp., *Caulerpa sertularioides* (S.G.Gmelin) M.Howe, and *Halimeda* sp.; n=9), or epilithic algae (aquarium with sterile sand and a rock with *Halimeda* sp. and diminutive bryopsidalean algal growth present; n=9), epilithic algae control (aquarium with sterile sand and an aquarium rock; n=1), or psammophytic algae control (aquarium with sterile sand only; n=1). Psammophytic algal taxa were selected based on their high abundance at the collection site, as well as their documentation as kleptoplast sources for *Plakobranchus* or other sacoglossan sea slugs (Hay et al. 1990, Maeda et al. 2012, Christa et al. 2013, 2014c). Live rock for the epilithic algae treatment was collected from Hunakai Beach, O‘ahu three weeks prior to the trial and was cultured in the large tank housing the aquaria to allow the diminutive green algae to increase in biomass. The experiment was allowed to run for 10 days until diatom growth began to affect water quality, and, therefore, compromise the health of the slugs and algae. Final photosynthetic yields of the sea slugs were measured upon termination

of the study using the same method as above. Kleptoplasts were sampled for a second time for comparison to those prior to the experiment; slugs were then released back to their collection site. Psammophytic and epilithic algae were sampled and sequenced for both *tuf*A and *rbc*L to populate a reference sequence framework for comparison to kleptoplast clone sequences.

2.3.5 Phylogenetic analyses. Forward and reverse sequence reads were edited, assembled into consensus sequences, and aligned with newly generated algal reference sequences and closely related GenBank sequences using MUSCLE in Geneious R6 or R7 (Biomatters, Auckland, New Zealand). Clone sequences were prone to single nucleotide polymorphisms; thus, those that originated from the same slug and clustered using both distance- and model-based methods were combined into a single consensus sequence. Seasonal datasets were combined and analyzed as one dataset in order to assess total diversity. Cloned sequences of the two gene regions were not concatenated because it could not be assumed that both sequences originated from the same algal individual. Jmodeltest 2.1.1 (Guindon and Gascuel 2003, Darriba et al. 2012) was used to determine the most appropriate models for phylogenetic analyses: the general time-reversible (GTR) model with gamma distribution and invariant sites (I+Γ). Maximum likelihood phylogenies were constructed using RAxML-HPC2 on XSEDE 8.1.11 (Stamatakis 2014), and the rapid bootstrap search was terminated using the MRE-based bootstrapping criterion (Pattengale et al. 2010). Bayesian inference phylogenies constructed using MrBayes on XSEDE 3.2.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) were run for 5×10^6 generations with a burnin value of 25%; congruence (standard deviation of split frequencies <0.05) was met for each analysis. Both RAxML and

MrBayes were accessed using the CIPRES Science Gateway (Miller et al. 2010). Algal species boundaries were inferred using the Geneious Species Delimitation plug-in (Masters et al. 2011) and were assessed using both the barcode gap (Hebert et al. 2003) and Rosenberg's p-value (Rosenberg 2007).

2.4 Results

2.4.1 *P. ocellatus* kleptoplast diversity

Since the individual gene datasets could not be concatenated, *tuf*A and *rbc*L gene trees were constructed separately. Using *tuf*A as a marker for algal species delineation, *P. ocellatus* from Hunakai was found to sequester chloroplasts from 11 species of bryopsidalean algae (Fig. 1.2), whereas the *rbc*L marker supported sequestration of 10 species of bryopsidalean algae in total (Fig. 1.3). One *tuf*A clone sequence was identified as *Pseudochlorodesmis* sp. ("*Siphonogramen*"), whereas *rbc*L did not detect this taxon; otherwise, the results for the two markers were comparable.

2.4.2 Sequestration preference trial. One slug mortality occurred in each of the psammophytic and epilithic treatments during the course of the experiment, reducing the design to eight replicates per treatment. Upon termination of the study, both control and psammophytic treatment slugs showed no obvious signs of newly sequestered chloroplasts (based on the absence of visible chlorophyll in the lamellae); conversely, slugs from the epilithic treatment did show evidence of sequestered chloroplasts. Presence/absence of newly sequestered chloroplasts was confirmed with photosynthetic yield measurements, in which the difference between the psammophytic and epilithic treatments was highly significant (t-value=-13.17, df=7, $p < 0.001$). Three of the slugs in the epilithic treatment did not yield kleptoplast sequences, presumably due to low concentration of kleptoplast DNA template. Phylogenetic

analyses of the five kleptoplast samples that did successfully amplify for *rbc*L suggested that *P. ocellatus* sequestered chloroplasts from six species within the genera *Caulerpa* (3 spp.), *Poropsis* (1 sp.), *Pseudochlorodesmis* (1 sp.), and *Rhipidosiphon* (1 sp.) during the trial (Fig. 1.3).

2.4.3 *Plakobranchus ocellatus* phylogeny. *Cox*I phylogenetic analyses demonstrated that *P. ocellatus* individuals from Hunakai, O‘ahu are part of a clade that includes previously published sequences from Maui (Hawai‘i), Australia, and the Philippines (Fig. 1.4). Interestingly, sequences from *P. ocellatus* from two sites on O‘ahu are not identical; the maximum p-distance between O‘ahu *Plakobranchus* individual sequences was 1.08%.

2.5 Discussion

2.5.1 *Plakobranchus ocellatus* kleptoplast diversity. *P. ocellatus* is a generalist algivore with up to six genera and 11 species of bryopsidalean algae used as kleptoplast sources by the Hawaiian population examined in this study. Both the *rbc*L and *tuf*A gene trees showed that eight of the detected species are new records of diminutive bryopsidalean green algae in the Hawaiian Islands in comparison to published records (Abbott and Huisman 2004): three *Caulerpa* spp. (*Caulerpa* Okamura clade), *Pseudochlorodesmis* sp. within the *Rhipilia/Rhipiliopsis* clade, two *Poropsis* spp., and two *Rhipidosiphon* spp. Draisma *et al.* (2014) demonstrated cryptic diversity within *Caulerpa ambigua*, which is further demonstrated here with two additional clades compared to those presented in their study. Both *Rhipidosiphon* species are considered new records, as they are molecularly distinct from *Rhipidosiphon javensis* published sequences – the only species recorded in the Hawaiian Islands.

All but one of these algae (*Halimeda*; n=1 clone sequence) are diminutive in size, suggesting that Hawaiian *P. ocellatus* prefers diminutive algae. While these results are similar to the findings of Maeda et al. (2012), they differ from those of Christa et al. (2014c), who concluded that sacoglossans that are long-term retainers of kleptoplasts, such as *P. ocellatus*, prefer either large bryopsidalean green algae (e.g. *Halimeda*, *Caulerpa*, *Avrainvillea*), *Acetabularia* (a dasycladalean green alga), or even *Vaucheria* (a siphonous xanthophyte alga).

2.5.2 *P. ocellatus* sequestration preference trial. *P. ocellatus*' preference for diminutive bryopsidalean green algae was further illustrated in the sequestration preference trials. When only large bryopsidalean genera, known to be kleptoplast sources for sacoglossans (Hay et al. 1990, Christa et al. 2013, 2014c), were offered, *P. ocellatus* did not sequester any new plastids. Additionally, slugs offered both large and diminutive taxa replenished their kleptoplasts, and molecular analyses of these kleptoplasts indicated that only chloroplasts from diminutive taxa were sequestered.

2.5.3 *P. ocellatus* phylogeny. Our results do not support *Plakobranchus* as a monotypic genus, a finding previously demonstrated by Krug et al. (2013). *P. ocellatus* from the Hawaiian Islands are members of a monophyletic, albeit diverse, group with a maximum p-distance of 2.34%. A minimum interspecific distance of 6.0% was proposed by Krug et al. (2013) for species delineation in the *P. ocellatus* complex, and a maximum intraspecific distance of 3.3% was demonstrated for the "*Plakobranchus* sp. 2" clade, which included Hawaiian sequences. However, these limits were defined using only two sequences from individuals collected on Maui; thus, increased representation from throughout the Hawaiian Islands may reveal more

complex biogeographic patterns (Cunningham and Collins, 1998, Toonen et al. 2011, Bowen et al. 2013).

As a generalist kleptoplastic sea slug, *P. ocellatus* provides a unique opportunity to explore both bryopsidalean algal diversity and a distinctive algal-herbivore interaction. *P. ocellatus*' preference for diminutive bryopsidalean taxa in Hawai'i suggests that biomechanical and/or physiological characteristics of the smaller siphonous algae may be driving this preference. Characteristics of the chloroplasts themselves may also play a significant role; it has been previously proposed that some bryopsidalean taxa, specifically *Halimeda, Caulerpa, Codium*, *Penicillus,* and *Avrainvillea*, produce chloroplasts more conducive to long-term retention by sacoglossans than others (Jensen 1997, Cruz et al. 2013, Christa et al. 2014c, de Vries et al. 2014, Pierce et al. 2015).

Algal thallus organization and characteristics may also influence *P. ocellatus*' sequestration preferences. Siphonous algae are characterized as either simple and uniaxial or complex and multiaxial (Vroom and Smith 2001, 2003, Graham et al. 2009). The diminutive taxa of this study are exclusively uniaxial (Graham et al. 2009). For example, the polyphyletic form genus *Pseudochlorodesmis* is phylogenetically related to more complexly organized genera (e.g. the *Rhipilia/Rhipiliopsis* clade), yet maintains a simple, uniaxial organization (Verbruggen et al. 2009). Although many siphonous genera are uniaxial as juveniles, they increase in complexity with maturity, as in the observed "*Pseudochlorodesmis*" stages of *Halimeda tuna* (J.Ellis and Solander) J.V.Lamouroux and *Botryodesmis* (Meinesz 1972, Abbott and Huisman 2004, Kraft 2007). Study results supporting the sequestration of chloroplasts from large, complex genera (e.g. Christa et al. 2013, 2014c) may in fact be reporting sequestration from juvenile algal individuals, which is an alternate explanation for

the findings of Christa et al. (2014c) that long-term retainers prefer large, complex, multiaxial algae. Additionally, thallus calcification is most likely not playing a role in limiting host selection. Only two of the genera recovered as kleptoplast sources in this study are calcified to any degree – *Halimeda* and *Rhipidosiphon*. If juvenile individuals are selected for as kleptoplast hosts, calcification can be avoided as juvenile, diminutive *Halimeda* and *Rhipidosiphon* plants are not yet calcified.

Several other factors may contribute to the preference results presented here. Some evidence suggests that preference may be due, in part, to incentives offered by algae, such as predator-deterring secondary compounds (Jensen 1997). As an example, the sacoglossan *Costasiella ocellifera* Simroth sequesters both chloroplasts and the predator deterrent avrainvilleol from its algal host, and, as a result, is able to deter predators (Hay et al. 1990). To date, no studies have assessed secondary chemistry of diminutive bryopsidalean algae, but they may also be offering similar anti-predatory compounds. Finally, preference could be attributed to substrate type and may be indicative of behavioral cues. Observations over a 24-h period demonstrated that *P. ocellatus* individuals at Hunakai Beach, O‘ahu take refuge at dusk and may be doing so under rocks or sand where epilithic, diminutive algae thrive (R. Wade, unpubl. data).

Studies of diminutive bryopsidalean algae are underrepresented in the literature, especially in comparison to taxa such as *Caulerpa* that represent some of the largest ulvophytes. This underrepresentation is likely due to their size and the resulting difficulty of their collection and identification. Additionally, these taxa have few distinct morphological characters and harbor substantial cryptic diversity (see Kooistra 2002, Verbruggen et al. 2009). Kleptoplasty by *P. ocellatus* presents an opportunity to explore diminutive

bryopsidalean diversity and, in this study, yielded eight new records at a single site in Hawai‘i. Future research combining an increased geographic scope with metabarcoding assessment of kleptoplast diversity and epiphytic and epilithic communities will provide a more thorough understanding of both bryopsidalean algal diversity and therefore *P. ocellatus* kleptoplasty throughout the tropical Pacific.

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2.8 Tables

Table 2.1 Gene region, primers, and cycling conditions for slug and kleptoplast analyses.

REGION	PRIMER	PRIMER SEQUENCE (5' → 3')	PROGRAM	SOURCE	GENBANK ACCESSIONS
*cox*I	coxFS	TTT CAA CAA ACC ATA ARG ATA TTG G	Initial denaturation for 95°C for 15 min, followed by 25 cycles of 94°C for 45 sec, 48°C for 45 sec, and 72°C for 90 sec, followed by a final extension of 72°C for 10 min.	Händeler et al. (2009)	KY012787-KY012790
	coxRS	TAY ACT TCW GGG TGW CCA AAA AAY CA			
*tuf*A	tufA_alg_up	ATG ATW ACN GGH GCN GCW CAA ATG G	Initial denaturation for 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for	Händeler et al. (2010)	KY205928-KY206007
	tufA_alg_do	TTG TTC KAA CAT AAA ATT GWG GTC			

			2 min, followed by a final extension of 72°C for 7 min.		
*rbc*L	rbcLF	AAA GCN GGK GTW AAA GAY TA	Initial denaturation for 95°C for 1 min, followed by 35 cycles of 94°C for 45 sec, 47°C for 45 sec, and 72°C for 90 sec, followed by a final extension of 72°C for 10 min.	Pierce et al. (2006)	KY062909-KY062985
	rbcLR	CCA WCG CAT ARA WGG TTG HGA			

2.9 Figures

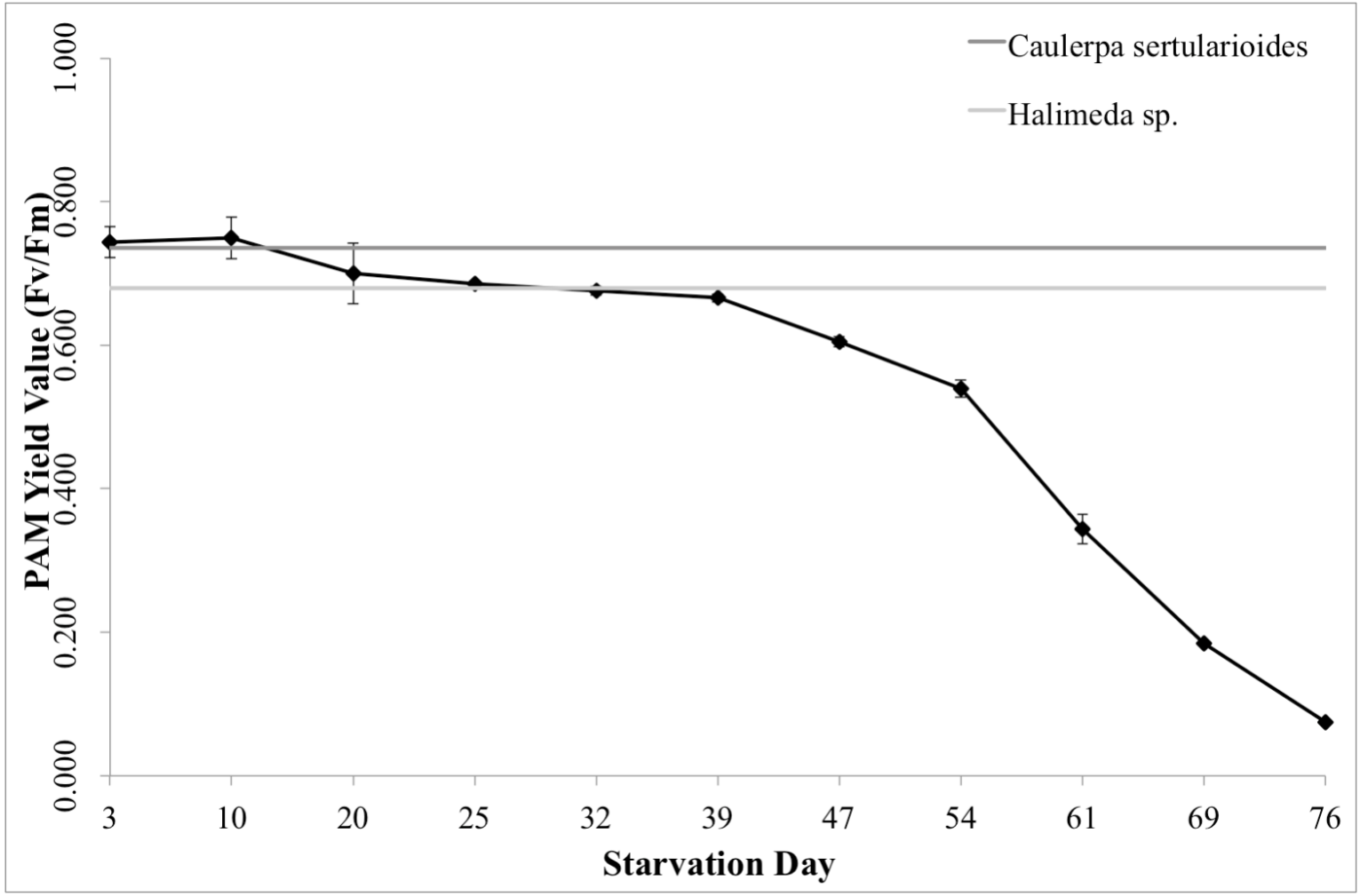
Caulerpa sertularioides
Halimeda sp.
PAM Yield Value (Fv/Fm)
0.000
0.200
0.400
0.600
0.800
1.000
3
10
20
25
32
39
47
54
61
69
76
Starvation Day

Figure 2.1 Decrease in kleptoplast photosynthetic yield (Fv/Fm) values of *Plakobranchus ocellatus'* sequestered chloroplasts during starvation period prior to sequestration experiment. Horizontal lines represent reference photosynthetic yield values for two healthy bryopsidalean algal species. Error bars = standard error.

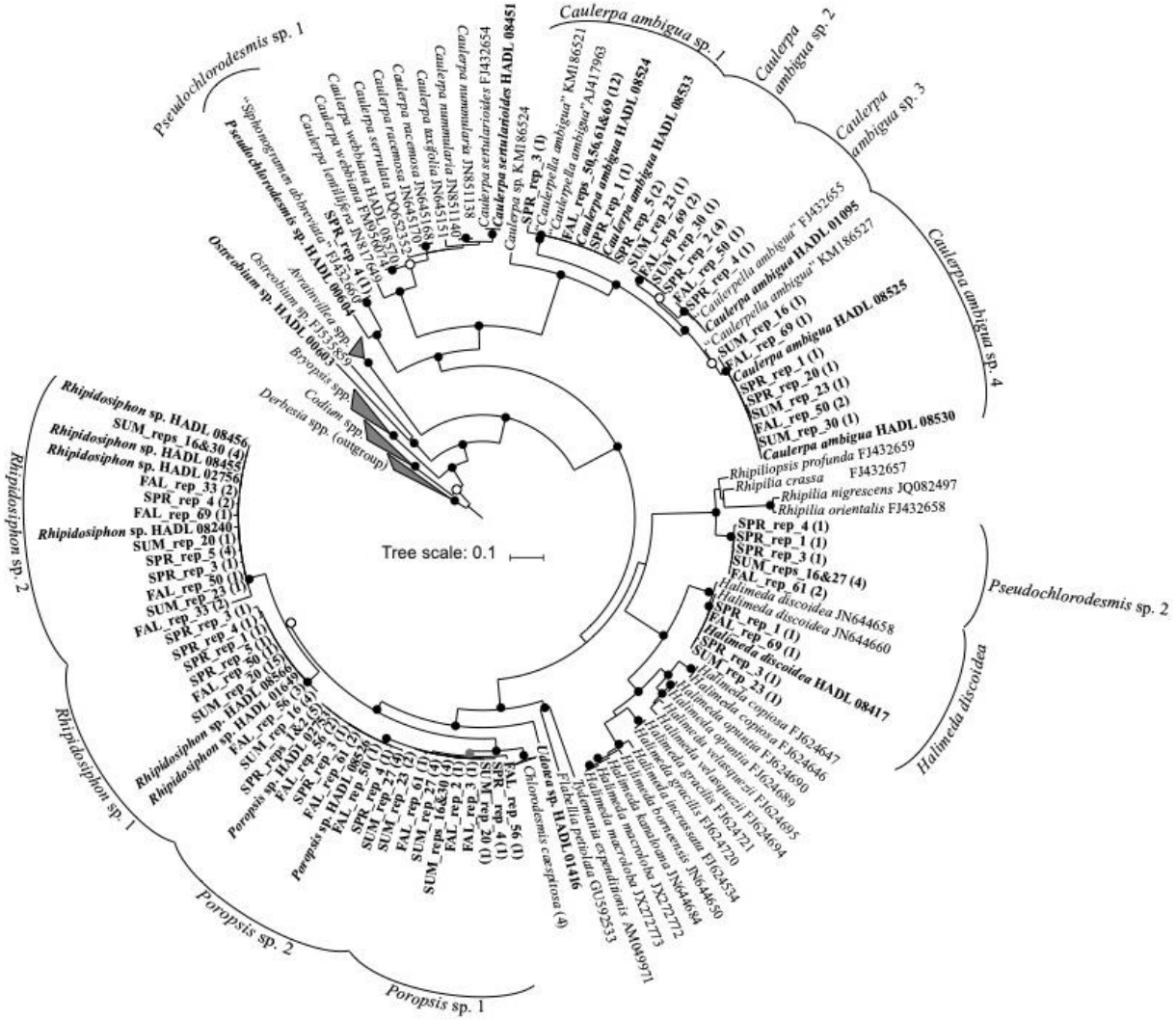
Pseudochlorodesmis sp. 1
Caulerpa ambigua sp. 1
Caulerpa ambigua sp. 2
Caulerpa ambigua sp. 3
Caulerpa ambigua sp. 4
Pseudochlorodesmis sp. 2
Halimeda discoidea
Rhipidosiphon sp. 2
Rhipidosiphon sp. 1
Poropsis sp. 2
Poropsis sp. 1
Tree scale: 0.1

Figure 2.2 Maximum likelihood estimated tufA phylogeny of Bryopsidales. Labels in bold represent sequences produced by this study; kleptoplast clone sequences denoted by season (SPR, SUM, FAL) and replicate identification number, followed by number of clone sequences represented in parentheses. Scale bar = substitutions per site. Node symbols represent support values of greater than 70%; black shapes represent bootstrap and posterior probability support, gray shapes represent bootstrap support only, and white shapes represent posterior probability support only.

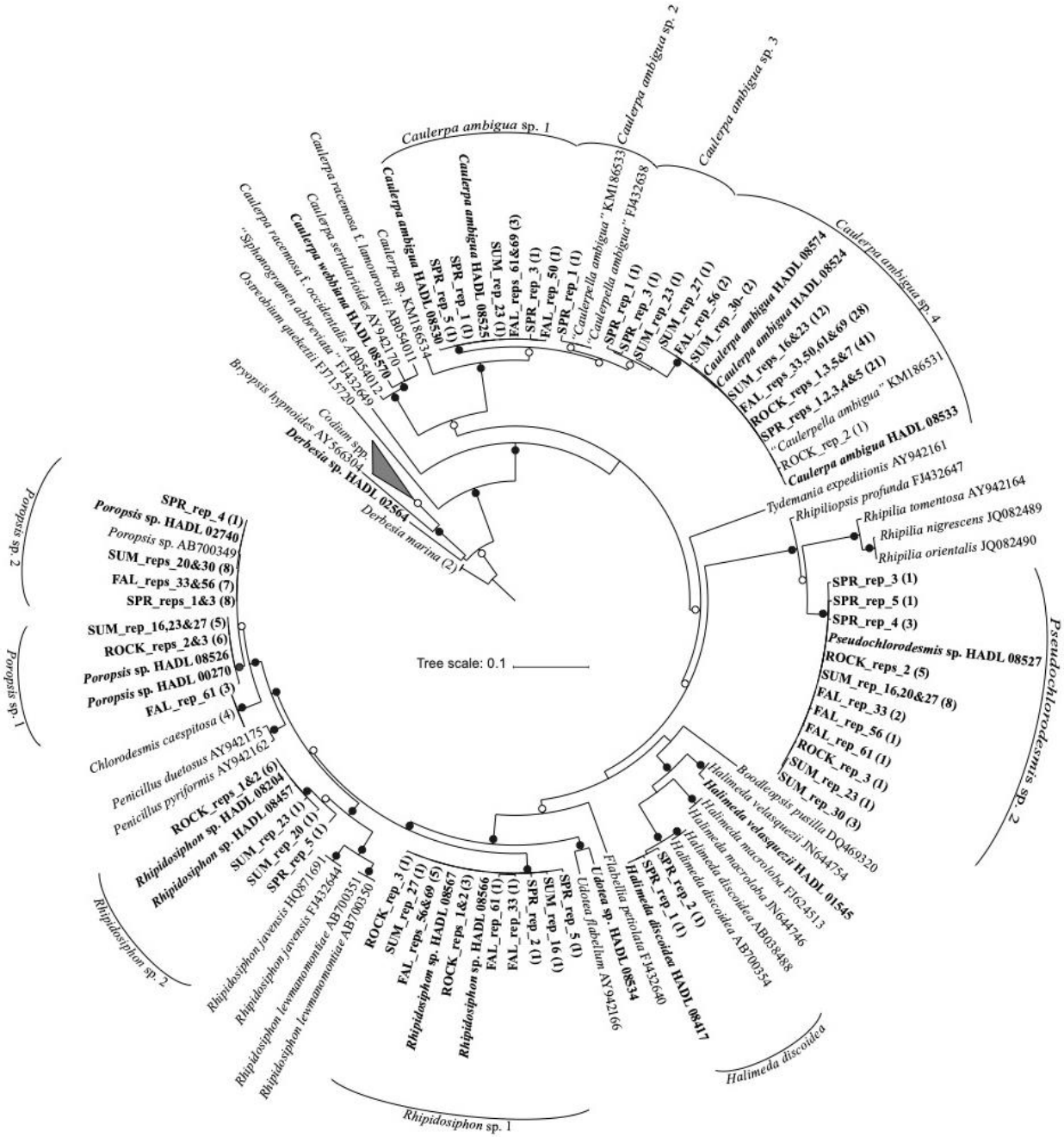
Caulerpa ambigua sp. 1
Caulerpa ambigua sp. 2
Caulerpa ambigua sp. 3
Caulerpa ambigua sp. 4
Caulerpa ambigua HADL 08525
SUM_rep_23 (1)
FAL_reps_61&69 (3)
SPR_rep_3 (1)
FAL_rep_50 (1)
SPR_rep_1 (1)
"Caulerpella ambigua" KM186533
"Caulerpella ambigua" FJ432638
SPR_rep_1 (1)
SPR_rep_3 (1)
SUM_rep_23 (1)
SUM_rep_27 (1)
FAL_rep_56 (2)
SUM_rep_30- (2)
Caulerpa ambigua HADL 08574
Caulerpa ambigua HADL 08524
SUM_reps_16&23 (12)
FAL_reps_33,50,61&69 (28)
ROCK_reps_1,3,5&7 (41)
SPR_reps_1,2,3,4&5 (21)
"Caulerpella ambigua" KM186531
Caulerpa ambigua HADL 08533
ROCK_rep_2 (1)
Caulerpa ambigua HADL 08530
SPR_rep_5 (1)
SPR_rep_1 (1)
Caulerpa sp. KM186534
Caulerpa lamourouxii AB054011
Caulerpa ambigua HADL 08570
Caulerpa serrularioides AY942170
Caulerpa racemosa f. lamourouxii AB054012
Caulerpa racemosa f. occidentalis AB054012
"Sphaerotagramen abbreviata" FJ432649
Caulerpa racemosa f. occidentalis AB054012
Osmundaria quekettii FJ715720
Bryopsis hypnoides AY566304
Codium spp.
Derbesia sp. HADL 02564
Derbesia marina (2)
Tydemania expeditionis AY942164
Rhipiliopsis profunda FJ432647
Rhipilia tomentosa AY942161
Rhipilia nigrescens JQ082489
Rhipilia orientalis JQ082490
SPR_rep_3 (1)
SPR_rep_5 (1)
SPR_rep_4 (3)
Pseudochlorodesmis sp. HADL 08527
ROCK_reps_2 (5)
SUM_rep_16,20&27 (8)
FAL_rep_33 (2)
FAL_rep_56 (1)
FAL_rep_61 (1)
ROCK_rep_3 (1)
SUM_rep_23 (1)
SUM_rep_30 (3)
Pseudochlorodesmis sp. 2
Boodleopsis pusilla DQ469320
Halimeda velasquezii JN644754
Halimeda velasquezii HADL 01545
Halimeda macroloba JN644746
Halimeda macroloba FJ624513
Halimeda discoidea AB038488
Halimeda discoidea AB700354
Halimeda discoidea HADL 08417
SPR_rep_2 (1)
SPR_rep_1 (1)
Halimeda discoidea HADL 08534
Filabellia petiolata FJ432640
Udotea flabellum AY942166
Udotea sp. HADL 08566
SUM_rep_5 (1)
SUM_rep_16 (1)
SPR_rep_2 (1)
FAL_rep_61 (1)
FAL_rep_33 (1)
Rhipidosiphon sp. HADL 08567
FAL_reps_5&69 (5)
SUM_rep_27 (1)
ROCK_rep_3 (1)
Rhipidosiphon sp. 1
Rhipidosiphon lewmanomontiae AB700350
Rhipidosiphon lewmanomontiae AB700351
Rhipidosiphon javensis FJ432644
Rhipidosiphon javensis HQ871691
Rhipidosiphon sp. 2
Rhipidosiphon sp. HADL 08457
SUM_rep_20 (1)
SPR_rep_5 (1)
Rhipidosiphon sp. HADL 08204
SUM_rep_23 (1)
ROCK_reps_1&2 (6)
Penicillus pyriformis AY942162
Penicillus duetosus AY942175
Chlorodesmis caespitosa (4)
FAL_rep_61 (3)
Poropsis sp. HADL 00270
Poropsis sp. HADL 08526
ROCK_reps_2&3 (6)
SUM_rep_16,23&27 (5)
Poropsis sp. 1
FAL_reps_33&56 (7)
SUM_reps_20&30 (8)
Poropsis sp. AB700349
Poropsis sp. HADL 02740
SPR_rep_4 (1)
Poropsis sp. 2
Tree scale: 0.1

Figure 2.3 Maximum likelihood estimated rbcL phylogeny of Bryopsidales. Labels in bold represent sequences produced by this study; kleptoplast clone sequences denoted by season or treatment in the sequestration preference trial (SPR, SUM, FAL, or ROCK, respectively) and replicate identification number, followed by number of clone sequences represented in parentheses. Node symbols represent support values of greater than 70%; black shapes represent bootstrap and posterior probability support, gray shapes represent bootstrap support only, and white shapes represent posterior probability support only. Scale bar = substitutions per site.

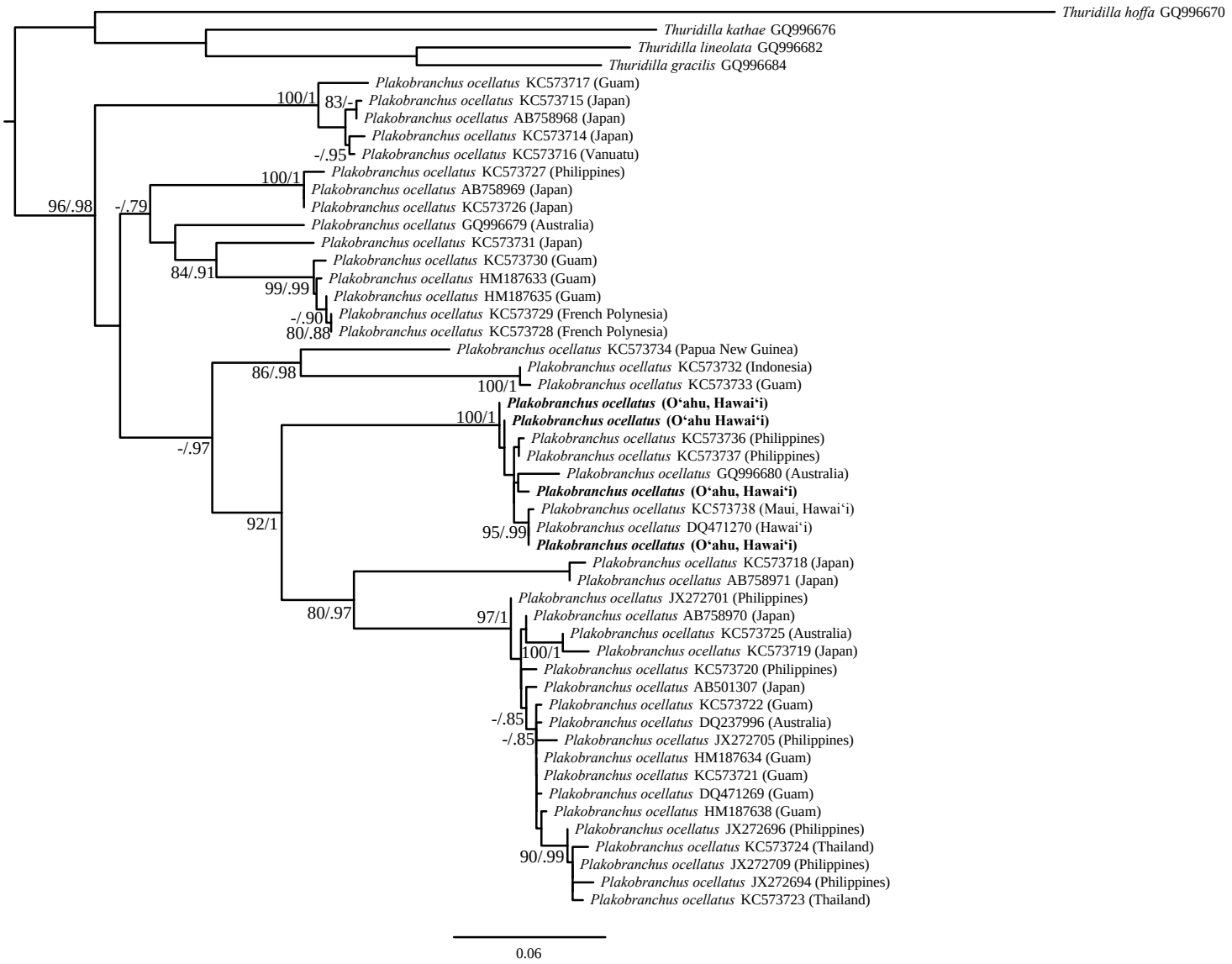
Thuridilla hoffa GQ996670
Thuridilla kathae GQ996676
Thuridilla lineolata GQ996682
Thuridilla gracilis GQ996684
Plakobranchus ocellatus KC573717 (Guam)
100/1
83/-
Plakobranchus ocellatus KC573715 (Japan)
Plakobranchus ocellatus AB758968 (Japan)
Plakobranchus ocellatus KC573714 (Japan)
-/.95
Plakobranchus ocellatus KC573716 (Vanuatu)
100/1
Plakobranchus ocellatus KC573727 (Philippines)
Plakobranchus ocellatus AB758969 (Japan)
96/.98
-/.79
Plakobranchus ocellatus KC573726 (Japan)
Plakobranchus ocellatus GQ996679 (Australia)
Plakobranchus ocellatus KC573731 (Japan)
Plakobranchus ocellatus KC573730 (Guam)
84/.91
Plakobranchus ocellatus HM187633 (Guam)
99/.99
Plakobranchus ocellatus HM187635 (Guam)
-/.90
Plakobranchus ocellatus KC573729 (French Polynesia)
80/.88
Plakobranchus ocellatus KC573728 (French Polynesia)
Plakobranchus ocellatus KC573734 (Papua New Guinea)
86/.98
Plakobranchus ocellatus KC573732 (Indonesia)
100/1
Plakobranchus ocellatus KC573733 (Guam)
***Plakobranchus ocellatus* (O'ahu, Hawai'i)**
100/1
***Plakobranchus ocellatus* (O'ahu Hawai'i)**
Plakobranchus ocellatus KC573736 (Philippines)
-/.97
Plakobranchus ocellatus KC573737 (Philippines)
Plakobranchus ocellatus GQ996680 (Australia)
***Plakobranchus ocellatus* (O'ahu, Hawai'i)**
Plakobranchus ocellatus KC573738 (Maui, Hawai'i)
92/1
95/.99
Plakobranchus ocellatus DQ471270 (Hawai'i)
***Plakobranchus ocellatus* (O'ahu, Hawai'i)**
Plakobranchus ocellatus KC573718 (Japan)
Plakobranchus ocellatus AB758971 (Japan)
Plakobranchus ocellatus JX272701 (Philippines)
80/.97
97/1
Plakobranchus ocellatus AB758970 (Japan)
Plakobranchus ocellatus KC573725 (Australia)
100/1
Plakobranchus ocellatus KC573719 (Japan)
Plakobranchus ocellatus KC573720 (Philippines)
Plakobranchus ocellatus AB501307 (Japan)
Plakobranchus ocellatus KC573722 (Guam)
-/.85
Plakobranchus ocellatus DQ237996 (Australia)
-/.85
Plakobranchus ocellatus JX272705 (Philippines)
Plakobranchus ocellatus HM187634 (Guam)
Plakobranchus ocellatus KC573721 (Guam)
Plakobranchus ocellatus DQ471269 (Guam)
Plakobranchus ocellatus HM187638 (Guam)
Plakobranchus ocellatus JX272696 (Philippines)
Plakobranchus ocellatus KC573724 (Thailand)
90/.99
Plakobranchus ocellatus JX272709 (Philippines)
Plakobranchus ocellatus JX272694 (Philippines)
Plakobranchus ocellatus KC573723 (Thailand)
0.06

Figure 2.4 Maximum likelihood coxI gene tree of *Plakobranchus ocellatus*. Support values represent maximum likelihood bootstrap values (first value) and Bayesian posterior probabilities (second value). Support less than 75% is represented by "-." Labels in bold represent sequences produced by this study. Scale bar = substitutions per site.

CHAPTER 3. Updating *Plakobranchus* cf. *ianthobapsus* (Gastropoda, Sacoglossa) host use: diverse algal-animal interactions revealed by NGS with implications for invasive species management

Rachael M. Wade & Alison R. Sherwood

3.1 Abstract

Sacoglossa, the "sap sucking" sea slugs, are highly specialized herbivores and the only metazoans that exhibit kleptoplasty, the sequestration and retention of chloroplasts from algae. *Plakobranchus* is one of the most generalistic herbivores within this order, with as many as 12 reported "algal host" (i.e. kleptoplast source) species. However, kleptoplast diversity studies conducted on *Plakobranchus* to date most likely underestimated the full diversity of kleptoplast sources within the studied populations due to limitations of the molecular techniques employed. Here, we apply a high throughput sequencing technique to assess kleptoplast diversity of *Plakobranchus* cf. *ianthobapsus*' from 10 sites across the Main Hawaiian Islands during winter and summer seasons. In so doing, we effectively used *P.* cf. *ianthobapsus* as a novel sampling tool to explore diminutive algal communities, including the current distribution of the invasive alga "*Avrainvillea amadelpha*." Our results show that *P.* cf. *ianthobapsus* sequesters chloroplasts from 23 algal species from across the siphonous green algal order Bryopsidales. We identified "*Avrainvillea amadelpha*" and *Codium edule* as new host species for *P.* cf. *ianthobapusus*, but their rarity among the data suggests they were most likely less preferential as hosts and were possibly utilized due to low abundance or unavailability of more preferable species, and therefore a response to starvation risk. Additionally, the identification of the highly invasive siphonous green alga "*A. amadelpha*" as a kleptoplast source provides new fine-scale range and distribution data for this problematic species. Overall kleptoplast diversity does not differ among sites, except in a coral-dominated, (i.e. not algal dominated) environment, suggesting that siphonous algal assemblages are common in algal-dominated ecosystems in the Hawaiian Islands. Diversity dissimilarity among seasons was recovered from the majority of sites sampled, supporting the need for seasonal data collection in algal diversity assessments. This case study using metabarcoding

of sacoglossan kleptoplasts provides deeper insights into these plant-animal interactions with a better understanding of host use than previous studies using traditional molecular methods and illustrates how algal diversity studies on the scale of plastids can have implications for understanding algal community structure and invasive species dynamics.

Keywords: *Avrainvillea*, Hawai‘i, metabarcoding, *rbc*L

3.2 Introduction

Order Sacoglossa comprises at least 300 species of sea slugs (WoRMS Editorial Board 2017). Sacoglossans are some of the most specialized herbivores in the world's oceans (Marín & Ros 2004) because the majority of species exclusively or preferentially feed on siphonous green algae (Jensen 1983, 1993, 1997; Marín & Ros 2004; Händeler et al. 2009; Christa et al. 2014). This specialization is demonstrated by the highly derived radular teeth that sacoglossans use to penetrate the cell walls of algae, and often show host-specific adaptations in shape or structure (Jensen 1983, 1993, 1997; Marín & Ros 2004; Händeler et al. 2009; Christa et al. 2014).

Sacoglossans are perhaps best known for sequestration and retention of algal chloroplasts, a phenomenon known as kleptoplasty (Kawaguti & Yamasu 1965). Within the Metazoa, this trait is only exhibited by Sacoglossa, but it is also expressed by ciliates and dinoflagellates (Pillet et al. 2011; Schoener & McManus 2012). Once cytoplasm enters the sacoglossan digestive system, chloroplasts are encased in host plasma membrane via phagocytosis by glandular tubules (Hirose 2005). The chloroplasts are then retained within the digestive gland without algal nuclei or other organelles for days to months and continue to photosynthesize and supply their host with photosynthate (Hinde & Smith 1975; Green et al. 2000; Casalduero & Muniain 2008; Yamamoto et al. 2013). Based on a lack of observable gut contents, it was originally proposed that long term retainers of kleptoplasts relied heavily

on these plastids for nutrition and growth (Hirose 2005); while this is true in some species, i.e. *Elysia chlorotica, crispata,* and *E. trisinuata* (Middlebrooks et al. 2011; Akimoto et al. 2004; Pierce et al. 2015) other species utilize kleptoplasty as more of a survival mechanism in response to starvation conditions than a primary source of nutrition (Jensen 1997; Marín & Ros 1992, 1993; Casalduero & Muniain 2008, Baumgartner et al. 2015).

Plakobranchus, although currently described as monotypic, is a species complex consisting of at least 10 genetically distinct species (Krug et al. 2013). This complex includes the first sacoglossan for which kleptoplasty was observed (Kawaguti 1941). Additionally, *Plakobranchus* uses a broad range of siphonous algae as "hosts" or kleptoplast sources; previous studies documented as many as 12 host species from the siphonous green algal orders Bryopsidales and Dasycladales (Maeda et al. 2012; Christa et al. 2013; Wade & Sherwood 2017). However, these studies have all used cloning and Sanger sequencing to assess kleptoplast diversity, and in the case of Wade & Sherwood (2017), rarefaction analyses suggested that the total diversity of kleptoplast source species was not recovered (R. Wade, unpublished data). Additionally, previous studies assessed single populations potentially representing different *Plakobranchus* species, and thus were not representative of kleptoplast diversity for the genus as a whole. To address these limitations, we sequenced multiplexed amplicons (e.g. metabarcoding) from *P.* cf. *ianthobapsus* (hereafter *Plakobranchus*) collected from across the Main Hawaiian Islands (MHI) on a Next Generation Sequencing (NGS) platform. The use of this technique allows a much deeper level of sequencing than Sanger sequencing (Loman et al. 2012), and thus can provide a more thorough exploration of the algal host species of *Plakobranchus*. This study's use of a kleptoplastic sea slug as a novel sampling tool allows not only better understanding of its use of algal host species sources, but

allows simultaneous exploration of siphonous green algal diversity, providing implications for better understanding algal community assemblage and its members.

3.3 Material and methods

3.3.1 Specimen collection

Between 3 and 12 with a median of 10 *Plakobranchus* specimens were collected haphazardly via snorkel from 10 sites across the MHI during Winter (Dec-Feb) 2014-2015 and Summer (Jun-July) 2015, for a total of 178 slug samples (Table 3.1). Slugs were transported back to the laboratory at the University of Hawai‘i at Mānoa campus and maintained by site in clean 500 mL watchglasses at ~25 °C with filtered, aerated seawater and room lighting for one week without food to allow algal cellular material to clear from their gut, and therefore eliminate plastid genetic signals other than those from sequestered kleptoplasts. Slugs were then frozen at -20 °C and subsequently stored at -80 °C.

3.3.2 Collection site characterization

Collection sites were characterized in two ways: by recording siphonous green algal diversity via qualitative snorkel surveys, and by using abiotic and biotic features provided by multilayer maps from the National Oceanographic and Atmospheric Administration (NOAA) National Centers for Coastal Ocean Science (NCCOS) Benthic Habitat project conducted in 2007 (Table 3.1; BAE Systems 2007). Qualitative surveys included haphazard searches for representative siphonous green algal species in the immediate area (~25 m^2) from which the slug specimens were collected. Representative algal specimens were collected, pressed, dried, and stored in the Sherwood laboratory herbarium (ARS09446-53). Sites were categorized by the number of siphonous algal species recovered; these categories were used to compare the diversity recovered using traditional snorkel sampling, versus that recovered from *Plakobranchus* kleptoplast metabarcode data.

Between 0 and 12 siphonous green algal species per site were present during the winter collections. Sites with <4 taxa present were categorized as Low (diversity), 4-10 as Medium, and >10 as High. This resulted in two sites being categorized as Low, seven as Medium, and one as High. The two sites with Low diversity – Kāneʻohe Bay Patch Reef 25, Oʻahu and Onekahakaha Beach Park, Hawaiʻi – each had a unique feature: Patch Reef 25 was the only site characterized by coral providing the majority of biological cover (rather than algae), and Onekahakaha had rocks and boulders providing geomorphological structure (rather than pavement or sand). The site with the highest siphonous green algal diversity – Hunakai Beach, Oʻahu - shared at least one characteristic (Reef Flat Zone) with all other sites and the same abiotic and biotic characteristics with six of the other sites, all of which were qualitatively categorized as having Medium diversity (Table 3.2).

3.3.3 Illumina library preparation and sequencing

Slug tissue was first homogenized with CTAB buffer in sterile WhirlPak® bags using a pestle before total genomic DNA was extracted from 200 µL of the tissue slurry with the OMEGA Biotek EZNA® Tissue DNA Kit (OMEGA Biotek, Norcross, GA, USA). Kleptoplast DNA was amplified for a portion of the *rbc*L (large subunit of the ribulose-bisphosphate carboxylase/oxygenase gene, 562 bp) chloroplast gene using adapted rbcLF and rbcLR primers (Pierce et al. 2006) and the amplification program used by Wade & Sherwood (2017). This *rbc*L barcode has been shown to delineate kleptoplast diversity and green algae in general to the species level (Leliaert et al. 2014; Wade & Sherwood 2017). Index primers were designed using a combination of the Kozich et al. (2013) and the "16S Metagenomic Sequencing Library Preparation: preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system" protocols (Illumina 2013a) (Appendix 1) and were annealed to amplicons in a second amplification step. Products from this second amplification were

purified using GE Healthcare Life Sciences Sera-Mag™ Carboxylate-Modified Magnetic SpeedBeads™ (Fisher Scientific, Pittsburgh, PA, USA) and quantified using a Life Technologies Qubit™ 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Libraries were normalized to equal concentration and pooled before submitting to the Hawai‘i Institute of Marine Biology (HIMB) Genetics Core Facility for sequencing. Libraries were run on a partial V3 MiSeq Illumina Sequencing Platform lane for 600 cycles.

3.3.4 Kleptoplast alpha diversity data analyses

The reverse reads were determined to be of low quality, as indicated by a sharp decline in phred score at lengths greater than ~120 bp; after conducting quality control filtering (see below), many samples were empty and only 19,932 sequences total (0.22% of the total) remained. Relaxing the length minimum to 175 bp (from 205 bp) increased the resulting dataset (to 2.3% of the total), but this length did not allow merging of the forward and reverse reads and did not produce confident phylogenetic OTU identifications alone. As a result, the forward and reverse reads were not merged and only the forward reads were used for downstream analyses. Because of the short length of the single read, quality control and analysis trials were conducted by assessing phylogenetic species recovered with increasing minimum Phred score to use the highest quality data possible without sacrificing diversity signal (Fig. 4.3.1), resulting in selection of a minimum average of 33 Phred score in the Trimmomatic sliding window (see below). This also removed phylogenetic noise (i.e. most likely false intraspecific divergence due to sequencing error). A summary of raw forward sequence data and the number of reads lost during quality control processing is provided in Table 3.3.

Complete pipeline commands can be found in Supplementary Information (Appendix 2). Raw forward reads were assessed for quality (using FastQC v. 0.11.2) and trimmed (using

Trimmomatic v. 0.36; Bolger et al. 2014) on the Galaxy platform (v. 0.67; Afghan *et al.* 2016). Trimmomatic parameters included HEADCROP (20 bp) to remove primer residues, trimming of the 3' end with SLIDING WINDOW (average of 4 bp, Q33), and MINLEN to remove short, uninformative reads (205 bp minimum, or 75% of maximum read length). Trimmed reads were then analyzed using MacQiime (v. 1.9.1; Caporaso et al. 2010). Reads were first demultiplexed and prepared for analysis (multiple_split_libraries_fastq.py). Chimeric sequences were identified (identify_chimeric_seqs.py) and removed (filter_fastq.py). Reads were binned into Operational Taxonomic Units (OTUs) (pick_otus.py) using the UCLUST algorithm (95%; Edgar 2010) and the most abundant representative sequences were used for downstream diversity analyses (pick_rep_set.py). Taxonomic identification of OTU representative sequences was achieved using BLAST against reference green algal *rbc*L previously generated (Wade & Sherwood 2017) and GenBank databases (assign_taxonomy.py; Altschul et al. 1999), as well as GenBank bacterial and sacoglossan genomes. OTU tables were then generated, from which singletons and contaminant sequences from extraction and amplification control samples were removed (filter_otus_from_otu_table.py). Remaining siphonous green algal OTU representative sequences were then exported for phylogenetic analysis using Geneious (v. 9.1.8; Biomatters, Auckland, NZ) to increase confidence in species level identification. Raw sequence reads were submitted to the GenBank Short Read Archive (BioProject ID PRJNA415221, accessions SAMN07819123-318).

Representative siphonous OTU reads were aligned with reference sequences using MUSCLE (Edgar 2004) in Geneious v. 10.2.3 (Biomatters, Auckland, NZ). The resulting alignment was used to reconstruct a Maximum Likelihood-estimated phylogeny using

RAxML-HPC2 on XSEDE 8.1.11 (Stamatakis 2014) via the CIPRES Gateway (Miller et al. 2010). Species limits for OTUs closely related phylogenetically were assessed using the Species Delimitation Plug-in (Masters et al. 2011) in Geneious and the resulting Barcode Gap (Hebert et al. 2003) and Rosenberg's *P*-value (Rosenberg 2007). OTUs were identified as bacteria (28.82% of representative OTU sequences), non-target organisms and/or non-target gene regions (<1.00%), *Plakobranchus* mitochondria (<1.00%), and siphonous green algae (14.03%). A large portion (56.22%) of the representative OTU sequences were unidentifiable; representative sequences did not return a BLAST hit using any reference databases (Table 3.3). OTUs corresponding to non-target organisms (i.e. terrestrial and obligate symbiotic algae, and angiosperms) and non-target gene regions (i.e. 23S ribosomal RNA) were most likely a result of MiSeq run carryover, in which samples from previous sequencing runs were still present on the platform during this study's sequencing run (Illumina 2013b). The remainder of analyses focused solely on target siphonous green algal OTUs.

3.3.5 Kleptoplast beta diversity analyses

A modified OTU table was used to conduct beta diversity analyses and construct a Principal Coordinates Analysis (PCoA) in MacQiime to compare kleptoplast diversity among seasons and collection sites using a jackknifed, unweighted Unique Fraction (UniFrac) metric (jackknifed_beta_diversity.py; Lozupone, & Knight; Vazquez-Baeza et al. 2013). Clustering was determined by assessing overlap, and therefore insignificant diversity dissimilarity, of the Inter-Quartile Range (IQR) ellipses produced by the jackknifing. Additionally, the beta-diversity PCoA was overlaid with a biplot to identify algal species most affecting dissimilarity (make_emperor.py; Vazquez-Baeza et al. 2013).

3.4 Results

3.4.1 Kleptoplast metabarcode alpha diversity

Plakobranchus from across the MHI sequestered chloroplasts from up to 26 species of siphonous green algae in the bryopsidalean suborders Bryopsidineae and Halimedineae (Fig. 4.3.2). Using the Barcode Gap and Rosenberg's *P*-value to delineate species, a more conservative 23 species were recovered due to the merging of several OTUs into one phylogenetic species e.g. *Pseudochlorodesmis* spp. 1 and 4, and *Chlorodesmis caespitosa* (Fig. 4.3.2). Interestingly, some kleptoplasts were determined to originate from *Codium*, the sole taxon recovered from the suborder Bryopsidineae, as well as *Avrainvillea*; neither have been previously reported as kleptoplast sources for *Plakobranchus*. These taxa were both of low abundance in the sequence dataset (2 and 58 sequences, respectively). *Codium* chloroplasts were recovered from one slug of the 10 collected during the summer collections from Anini Beach Park, Kauaʻi, *Avrainvillea* was recovered from Patch Reef 25 in Kāneʻohe Bay, Oʻahu (1 slug of 10 in winter), Makai Pier, Oʻahu (3 slugs of 10 in winter, 1 slug of 10 in summer), Maunalua Bay via Paiko Rd, Oʻahu (1 slug of 10 in winter, 3 slugs of 10 in summer), Anahola Beach Park (1 slug of 10 in summer), Kauaʻi, and Anini Beach Park, Kauaʻi (1 slug of 10 in summer) (Fig. 4.3.3).

3.4.2 Kleptoplast metabarcode beta diversity

Samples from Onekahakaha Beach Park, Hawaiʻi and Maunalua Bay via Paiko Road, Oʻahu sequenced poorly (44 and 348 total sequences, respectively) and were removed from all beta diversity analyses in order to include rigorous rarefaction (both among seasons within sites, and among sites analyses). Data from the summer collections from Anini Beach, Kauaʻi and the winter collections from Lahaina Waterfront, Maui and Makai Pier, Oʻahu were also removed from beta diversity analyses among season within sites due to poor sequencing. Beta

diversity analyses suggested diversity dissimilarity among seasons within all sites, except for the Lāʻie "bathtub" – a small, protected, manmade lagoon that backs a residential area (Fig. 4.3.4a). Five clusters resulted from the PCoA assessment seasonal diversity by site: the summer collection from Kāneʻohe Bay Patch Reef 25 alone, the winter collection from Kāneʻohe Bay Patch Reef 25 alone, the summer collections from Lahaina Waterfront, Maui alone, the winter collections from Hunakai Beach, Oʻahu alone, and a large cluster containing the remaining seasonal collections by site (Fig. 4.3.4a). Weak dissimilarity of the Hekili Point, Maui summer collections and the Anahola Beach, Kauaʻi winter collections was supported; these data points' IQR ellipses slightly overlapped with that of the Makai Pier summer collections. Species responsible for the seasonal diversity among sites dissimilarity were primarily members of the *Caulerpa ambigua* complex (species 1, 5, and 6), *Halimeda discoidea*, *Poropsis* sp. 2, and *Rhipidosiphon* sp. 3. Interestingly, *Halimeda discoidea* was identified from *Plakobranchus* kleptoplasts from all sites, except Onekahakaha Beach Park, Hawaiʻi and Kāneʻohe Bay Patch Reef 25 – the two sites categorized as having Low diversity.

Seasonality alone was not responsible for dissimilarity among sites; when seasonality was removed and beta diversity analyses were conducted among sites only, kleptoplast diversity similarity was supported among all sites, except for those sampled from Patch Reef 25 in Kāneʻohe Bay, Oʻahu (Fig. 4.3.5a). Dissimilarity of both Maui sites from the large cluster was weakly supported. Comparable to the dissimilarity supported with seasonality by site, members of the *Caulerpa ambigua* complex (spp. 1 and 6) and *Halimeda discoidea* strongly drove the site diversity dissimilarity; *Poropsis* sp. 1 and *Pseudochlorodemis* sp. 2 also contributed to the dissimilarity among sites when seasonality was ignored. Beta diversity analyses both with and without seasonal variability suggested little diversity structure based

on island location (Figs. 3.4b and 3.5b), although weak dissimilarity of one or both Maui sites was supported.

3.5 Discussion

3.5.1 Overall kleptoplast diversity

The number of species detected here is considered conservative, as the species delimitation analyses resulted in the merging of multiple OTUs into less phylogenetic species and the number of OTUs recovered before phylogenetic assignment was considerably higher (42 vs. 23, Table 3.1). Even with this conservative approach and the rigorous quality control filtering, overall kleptoplast diversity recovered from *Plakobranchus* cf. *ianthobapsus* identified in this study was more than double the diversity previously reported in prior studies (Wade & Sherwood 2017), and nearly triple that from others (Maeda et al. 2008). This outcome is likely a result of the power of the sequencing platform and metabarcoding methods employed in this study and demonstrates the utility of these techniques in community diversity studies on this fine of scale (i.e. plastids). However, the increased diversity recovered here could also be a reflection of increased algal community diversity and/or the study of a different *Plakobranchus* sp. Despite this, this study demonstrates that *Plakobranchus* may be more specialized than the overall diversity presented here suggests. In Hawai‘i, 19 species of *Caulerpa* (Abbott & Huisman 2004; Wade & Sherwood 2017), and eight species of *Halimeda* (Abbott & Huisman 2004; Verbruggen et al. 2006) are recorded, of which 15 and 5 species, respectively, are recorded from intertidal to shallow subtidal (<25m) environments; however, *Plakobranchus* only uses one or a few from each genus as kleptoplast sources. This suggests that despite overall generalistic host use throughout the suborder Halimedineae, *Plakobranchus* may have specialized associations with each of the species recovered, and therefore may have a much more complex relationship with its algal hosts than previously

described. Specifically, this study demonstrates that calcification (e.g. *Halimeda*), anti-herbivore chemical defenses (e.g. *Avrainvillea, Caulerpa*), and thallus construction (i.e. uniaxial vs. multiaxial; e.g. *Caulerpa* and *Halimeda*, respectively) are not limiting *Plakobranchus*' host selection. Regardless of being overall diminutive in stature, perhaps it is actually siphon diameter or other localized fine-scale phenomena that are scaled to the size of the slugs' teeth and radula that are more important drivers of host selection.

3.5.2 Algal diversity among sites

Little difference was identified in algal diversity among sites at both the site and island levels. This was surprising considering that there are recognized barriers for fish, sessile invertebrates, and marine mammals in the Hawaiian Islands (Toonen et al. 2011). Similar barriers could be applicable to algae (particularly siphonous green algae) which also reproduce by broadcast spawning, but interestingly those barriers were not detected in this study. Among-site differences in algal diversity might also be attributed to intraspecific host specialization by *Plakobranchus* in response to recent population divergence; however, *Plakobranchus* in the MHI represents a single genotype (Appendix 3). One factor that does seem to be affecting the diversity among sites is the dominant biological cover – Kāneʻohe Bay Patch Reef 25, Oʻahu, which solely comprises one of the two clusters recovered from among-site beta diversity analyses (Fig. 4.3.4a) is the only site sampled that is coral dominated (10-<50% cover). All other sites, and thus those that comprise the other cluster, are algal dominated (10-100% cover). Additionally, the absence of *Halimeda discoidea* at the patch reef site (determined both qualitatively and by its absence in recovered kleptoplast diversity) may be an important driver of diversity differences among sites. Similarly, the presence and absence of diminutive species are influencing overall algal diversity dissimilarity between sites (Fig. 4.3.4a). These factors – dominant biological cover and/or

presence/absence of macrophytes and/or diminutive algal species - may be indicative of additional abiotic and/or biotic differences at the substrate level that are influencing the algal community at specific sites, including influences of overall community composition, nutrient inputs, or grazing pressure, etc. (*viz.* the relative-dominance paradigm) (Littler & Littler 1984; Littler et al. 1991).

3.5.3 Ecologically insignificant host use & invasive species detection

Perhaps the most interesting and unexpected outcome of this study is the detection of *Avrainvillea* and *Codium* kleptoplasts. Two species of *Avrainvillea* have been reported in the MHI: *"A. amadelpha*", which was first recorded in 1981 in the western shore's intertidal zone of O'ahu (Brostoff 1989), and whose species-level identification is currently under debate (Wade et al. 2015), and *A. erecta*, which was first recorded in October 2015 at 12-15 m depth near an O'ahu south shore harbor (Wade et al. in revision). There is also a single isolated record of "*A. amadelpha*" off Kaua'i's south shore (Smith et al. 2002), but subsequent inspections of this site in 2014, 2015, 2017, and 2018 did not confirm the persistence of the population (R. Wade, unpublished data). *Avrainvillea* in Hawai'i has few documented herbivores (literature reports include the parrotfish *Calotomus carolinus*, surgeonfish *Acanthurus nigricauda* and *Naso lituratus*, McCauley et al. 2010, and the collector urchin *Tripneustes gratilla*, Van Heukelem 2016), which may be due to its probable recent introduction and the documented anti-herbivory chemical profile of members of the genus (Sun et al. 1983). However, these herbivory accounts were based on changes in algal weights, not first-hand observations or gut contents. Additionally, observations of herbivores taking bites out of *Avrainvillea* and promptly spitting it out have been made in the field (S. Chulakote, pers. comm.). Thus, these herbivory accounts are somewhat inconclusive. Our identification of *Plakobranchus* kleptoplasts originating from "*A. amadelpha*" supports

Plakobranchus as a conclusive herbivore of this invasive alga in Hawai‘i due to its integration of *Avrainvillea* kleptoplasts. While there are no data to date supporting *Plakobranchus* as having an effect on the populations of “*A. amadelpha*”, the potential effects of herbivory on this species should be further investigated, particularly in environments recently invaded by the alga that are also home to *Plakobranchus* (Trowbridge & Todd 2001; Trowbridge 2002).

Additionally, recovery of “*A. amadelpha*” from *Plakobranchus* kleptoplasts provides new distribution information for this invasive alga. Slugs collected from Anahola and Anini Beach Parks on the eastern and northern shores of Kaua‘i, respectively, were found to have sequestered chloroplasts from the invasive alga (Fig. 4.3.3). Thus, “*A. amadelpha*” has indeed spread beyond the island of O‘ahu and is established outside the single Kaua‘i south shore population reported by Smith et al. (2002). In addition, slugs collected from Patch Reef 25 in Kāne‘ohe Bay, O‘ahu were also found to have sequestered chloroplasts from “*A. amadelpha*” (Fig. 5). Regular surveys for invasive algae have been conducted by the Hawai‘i State Department of Land and Natural Resources Division of Aquatic Resources (DLNR DAR) in Kāne‘ohe Bay and have only located “*A. amadelpha*” in the northern part of the bay, not the central part of the bay where Patch Reef 25 is located (C. Gewecke & B. Nielsen, personal communication). While the data presented here only provide a small range expansion, they further illustrate the point made by Smith et al. (2002) that “*A. amadelpha*” can persist as a cryptic and diminutive alga in Hawai‘i, contributing to its presumed absence and the likelihood of it being overlooked when traditional surveying methods are used. Confirmed herbivory by *Plakobranchus* on the invasive presents an ideal opportunity to test the proposal that *Plakobranchus* can be used as a novel sampling tool to explore cryptic and diminutive bryopsidalean algal diversity (Wade & Sherwood 2017), in this case using *Plakobranchus*

populations in Hawai‘i to track “*A. amadelpha*.” However, it should be emphasized that *Plakobranchus* did not sequester chloroplasts from “*A. amadelpha*” at every site that was documented to host both the slug and the invasive alga – “*A. amadelpha*” was not recovered from slugs collected from Hunakai Beach, O‘ahu in this study or in the Wade & Sherwood (2017) surveys, despite the alga’s presence at this site. Given that this site had the highest diversity recovered from the qualitative surveys, it is possible that *Plakobranchus* ignored the alga and grazed on preferred species, e.g. diminutive, halimedinean species (Wade & Sherwood 2017).

The detection of *Codium* kleptoplasts also presents interesting implications for host selection of *Plakobranchus*. First, this is the first record of *Codium* as a chloroplast source for *Plakobranchus.* Second, this is the first alga to be identified from *Plakobranchus* kleptoplasts outside of the suborder Halimedineae of order Bryopsidales. The Bryopsidineae, which includes *Codium*, and the Halimedineae, differ substantially in terms of thallus construction (primarily uniaxial versus primarily multiaxial), cell wall composition (primarily mannan versus primarily xylan; Hillis-Colinvaux 1984), reproduction (non-holocarpic versus holocarpic; Hillis-Colinvaux 1984; Graham et al. 2009; Niklas & Kutschera 2010), which can result in significant loss of adult individuals and therefore reduced abundance in the latter (see Vroom et al. 2003), and plastid specialization (homoplastic versus heteroplastic; Hillis-Colinvaux 1984; Graham *et al.* 2009). While *Codium* is the one multiaxial exception in the Bryopsidineae (Verbruggen et al. 2009), it exhibits the other bryopsidinean characteristics described above. Because of these differences, *Codium* is less appropriate as a host for *Plakobranchus*; more specifically, *Plakobranchus* tooth shape and function is likely better suited for feeding on Halimedineae taxa with smaller cell wall diameter (Marín & Ros 2004;

Hirose 2005). With the unexpected recovery of both *Avrainvillea* and *Codium,* greater kleptoplast diversity recovery in this study should be carefully considered, as the recovery of both species may be response to low abundance or diversity of desirable algae, and therefore in response to starvation, and ecologically insignificant as kleptoplast host species for *Plakobranchus.*

3.5.4 Implications of kleptoplast diversity for retention and longevity

Plakobranchus is not the sole sacoglossan that demonstrates such speciose host use; *Elysia crispata* (=*E. clarki*, Krug et al. 2016) and *E. viridis* are quite generalistic with recorded host species of 11 and four host genera, respectively from across multiple orders of green algae, including members of the non-siphonous and multicellular, but similarly multinucleate and large celled order Cladophorales (Curtis et al. 2006, Pierce et al. 2006, Christa et al. 2014, Middlebrooks et al. 2014). These host species counts are based on observations and/or feeding manipulations (*E. viridis*) and Sanger sequencing of cloned amplicons (*E. crispata*), and thus, the real number is most likely much larger, as in the case of *Plakobranchus* demonstrated here. Interestingly, all of these sacoglossans are long-term retainers of sequestered chloroplasts. However, their host use is quite varied; while *Plakobranchus* sequesters chloroplasts primarily from species from order Bryopsidales, suborder Halimedineae or order Dasycladales, *E. viridis* primarily utilizes species of the orders Cladophorales or Bryopsidales, suborder Bryopsidineae. *Elysia crispata* shares host species with both *Plakobranchus* and *E. timida* (i.e. *Halimeda*), but also uses the bryopsidinean *Bryopsis* and *Derbesia* as juveniles (Curtis et al. 2005), the halimedinean *Penicillus,* and the dasycladalean *Acetabularia* (Curtis et al. 2005, 2006). Clearly, there is not a single prescription in terms of

host use for kleptoplast maintenance and longevity, and therefore the drivers of long-term retention are likely to be complex.

3.6 Conclusions

The combination of kleptoplast metabarcoding and Next Generation Sequencing allows a deeper understanding of sacoglossan host use to be explored than has not been previously possible with Sanger sequencing and cloning technologies and provides a novel method for exploring algal communities and assessing an invasive algal species' ecology and distribution. Continued use of deep sequencing techniques may be key to early detection of range expansion and continued monitoring of Hawai‘i's invasive *Avrainvillea*.

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3.10 Tables

Table 3.1. *Plakobranchus* and algal collection and site information.

Island	Site	Latitude	Longitude	Winter Date Sampled	# Slugs Sampled	Summer Date Sampled	# Slugs Sampled
Hawai‘i	Onekahakaha Beach Park	22.738295	-159.038652	22-Dec-14	3	19-Jun-15	10
Kaua‘i	Anahola Beach Park	22.14645	-159.298501	19-Dec-14	10	12-Jun-15	10
Kaua‘i	Anini Beach Park	22.224216	-159.446464	19-Dec-14	10	12-Jun-15	10
Maui	Hekili Pt	20.809093	-156.622883	1-Feb-15	10	3-Jul-15	6
Maui	Lahaina Waterfront	20.865004	-156.67394	1-Feb-15	10	3-Jul-15	10
O‘ahu	Hunakai Beach Park	21.262764	-157.783565	7-Dec-14	12	13-Jul-15	10
O‘ahu	Kāne‘ohe Bay Patch Reef 25	21.460839	-157.823121	10-Feb-15	6	7-Jul-15	10
O‘ahu	Laie "Bath tub"	21.635759	-157.918672	8-Jan-15	11	1-Jul-15	10
O‘ahu	Makai Pier	21.262764	-157.783565	7-Dec-14	10	1-Jul-15	10
O‘ahu	Maunalua Bay via Paiko Rd	21.281105	-157.728191	25-Jan-15	10	1-Jul-15	10

Table 3.2. Collection site characterization, including abiotic and biotic data and a comparison of qualitative algal survey data and kleptoplast metabarcode data.

Site	Zone	Geo-morphological Structure	Biological Cover	Siphonous Algal Diversity via Qualitative Surveys	Total spp.	Qualitative Algal Diversity Categorization	Siphonous Algal Diversity via Kleptoplast Metabarcoding	Total spp.
Onekahakaha Beach Park	Reef Flat	Rock & Boulder	Macroalgae 10-<50%	*Codium edule, Codium* sp.	2	Low	*Caulerpa ambigua* sp. 6, *Caulerpa webbiana*, *Chlorodesmis caespitosa*, *Poropsis* sp. 2, *Pseudochlorodesmis* sp. 2, *Pseudochlorodesmis* sp. 3	6

Anahola Beach Park	Reef Flat	Pavement	Macroalgae 10-<50%	*Caulerpa racemosa*, *Caulerpa webbiana*, *Halimeda* sp., *Bornetella sphaerica*, *Neomeris* sp.	5	Med	*Avrainvillea* sp., *Caulerpa ambigua* spp. 1-6, *Caulerpa webbiana*, *Chlorodesmis caespitosa*, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (5), *Rhipidosiphon* sp. 2 & 3	19
Anini Beach Park	Reef Flat	Pavement	Turf 50-<90%	*Caulerpa serrulata*, *Halimeda* sp., *Codium arabicum*,	5	Med	*Avrainvillea* sp., *Caulerpa ambigua* spp. 1-6, *Caulerpa lentillifera*, *Caulerpa webbiana*,	22

				Bornetella sphaerica, *Neomeris* sp.			*Chlorodesmis caespitosa*, *Codium edule*, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (5), *Rhipidosiphon* sp. 2 & 3, *Udoteaceae* sp.	
Hekili Pt	Reef Flat	Pavement	Macroalgae 10-<50%	*Caulerpa serrulata*, *Halimeda* spp. (2), *Neomeris* sp.	4	Med	*Caulerpa ambigua* spp. 1-6, *Caulerpa webbiana*, *Chlorodesmis caespitosa*, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (6), *Rhipidosiphon* sp. 2 & 3, *Udoteaceae* sp.	20

Lahaina Waterfront	Reef Flat	Pavement, Sand	Macroalgae 10-<50, Uncolonized 90-100%	*Caulerpa serrulata*, *Codium edule*, *Halimeda* spp. (2), *Neomeris* sp.	5	Med	*Caulerpa ambigua* spp. 1-6, *Chlorodesmis caespitosa*, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (6), *Rhipidosiphon* sp. 2 & 3, *Udoteaceae* sp.	19
Hunakai Beach Park	Reef Flat	Pavement	Macroalgae 10-<50%	*Avrainvillea* sp., *Caulerpa ambigua*, *Caulerpa racemosa*, *Caulerpa webbiana*, *Codium edule*, *Halimeda* spp. (2), *Rhipidosiphon*	12	High	*Caulerpa ambigua* spp. 1-6, *Caulerpa webbiana*, *Chlorodesmis caespitosa*, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (6), *Rhipidosiphon* spp. 1-3.	20

				spp. (2), *Bornetella sphaerica*, *Neomeris* sp., *Parvocaulis parvula*				
Kāneʻohe Bay Patch Reef 25	Reef Flat	Individual Patch Reef	Coral 10-50%		0	Low	*Avrainvillea* sp., *Caulerpa ambigua* spp. 1 & 4, *Chlorodesmis caespitosa*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (6), *Rhipidosiphon* sp. 2 & 3, *Udoteaceae* sp.	14
Laie "Bath tub"	Reef Flat	Sand	Uncolonized 90-100%	*Halimeda* sp., *Rhipidosiphon* sp., *Bornetella sphaerica*, *Neomeris* sp.	4	Med	*Caulerpa ambigua* spp. 1-6, *Caulerpa webbiana*, *Chlorodesmis caespitosa*, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. (6), *Rhipidosiphon* sp. 2 & 3	19

Makai Pier	Reef Flat	Pavement	Macroalgae 10-<50%	*Caulerpa serrulata*, *Halimeda* sp., *Bornetella sphaerica*, *Neomeris* sp.	4	Med	*Avrainvillea* sp., *Caulerpa ambigua* spp. 1-5, *Chlorodesmis caespitosa*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* spp. 2, 3, & 5, *Rhipidosiphon* sp. 2 & 3	14
Maunalua Bay via Paiko Rd	Reef Flat	Pavement	Macroalgae 10-100%	*Avrainvillea* sp., *Caulerpa sertularioides*, *Halimeda* sp., *Rhipidosiphon* sp., *Bornetella sphaerica*	5	Med	*Avrainvillea* sp., *Caulerpa ambigua* spp. 1, 2, 4, 6, *Halimeda discoidea*, *Poropsis* sp. 1, 2, *Pseudochlorodesmis* sp. 3, *Rhipidosiphon* spp. 1, 2, 3	12

Table 3.3. Reads remaining after each step of quality control filtering and data analysis using the QIIME pipeline.

		% Raw Data	% Filtered Data	# Reads	# OTUs
Pipeline step	Raw sequences	100		8,780,777	
	Trim & Quality control filtering	8.70		764,005	
	Chimera & poorly sequenced sample removal	8.53		749,362	27,863
	Denoising (removal of singletons)	8.39		737,014	15,515
	Decontamination (removal of control sample OTUs)	6.35		556,939	15,058
OTU sequences by higher taxonomic groups	Bacteria	1.82	28.82	160,517	5,804
	No BLAST hit	3.57	56.24	313,201	9,010
	Non-target organisms/gene regions	<1	<1	51	15
	Plakobranchus mtDNA	<1	<1	4.885	1

	Siphonous green algae	<1	14.07	78,357	227
	Siphonous green algae OTUs ≥100 reads	<1	13.75	76,578	42

3.11 Figures

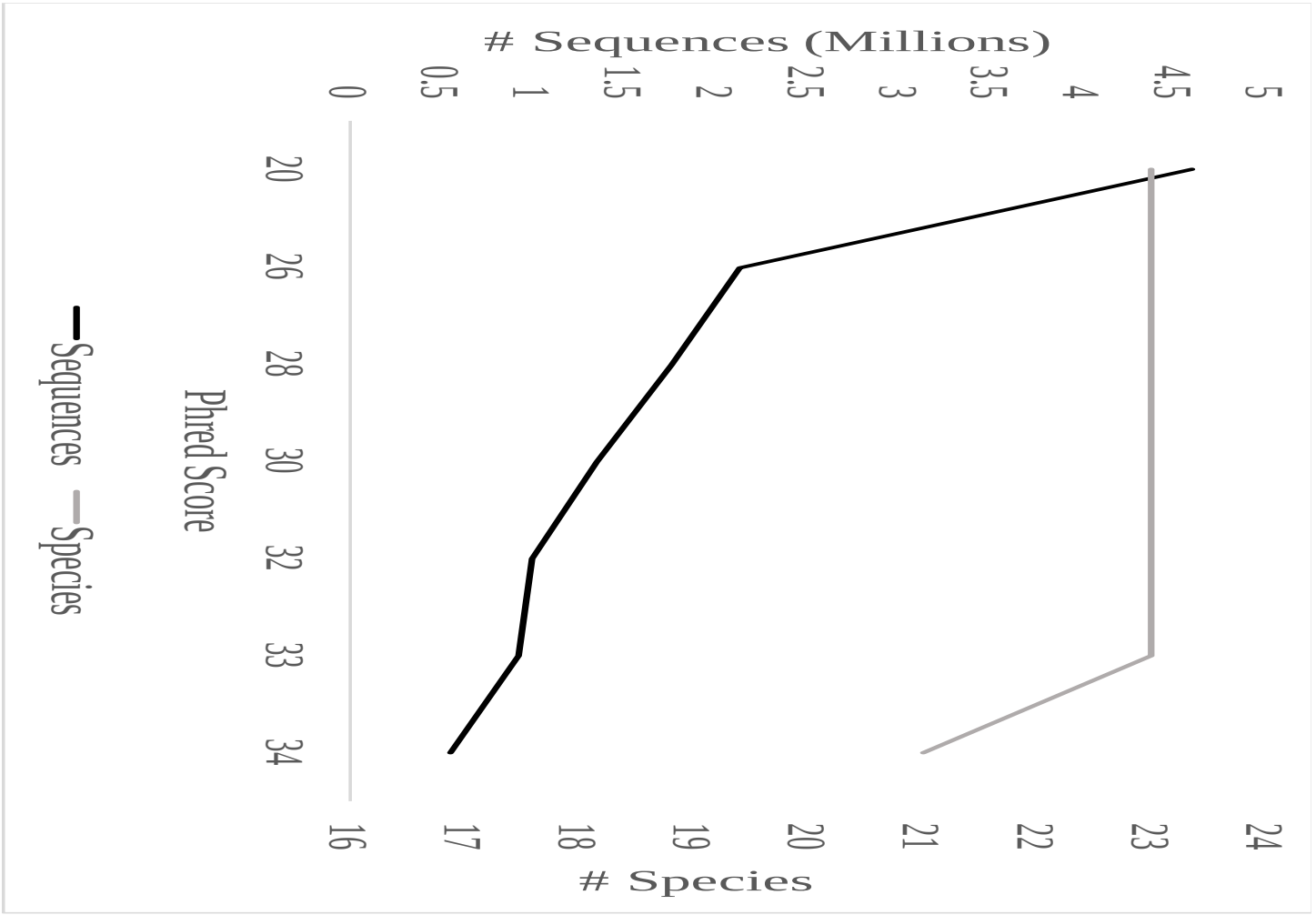

Sequences (Millions)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
20
26
28
30
32
33
34
Phred Score
Sequences
Species
16
17
18
19
20
21
22
23
24
Species

Figure 3.1. Relationship between increase in Phred score for sequencing quality control filtering and decrease in both number of sequences and diversity signal (i.e. number of phylogenetic species recovered).

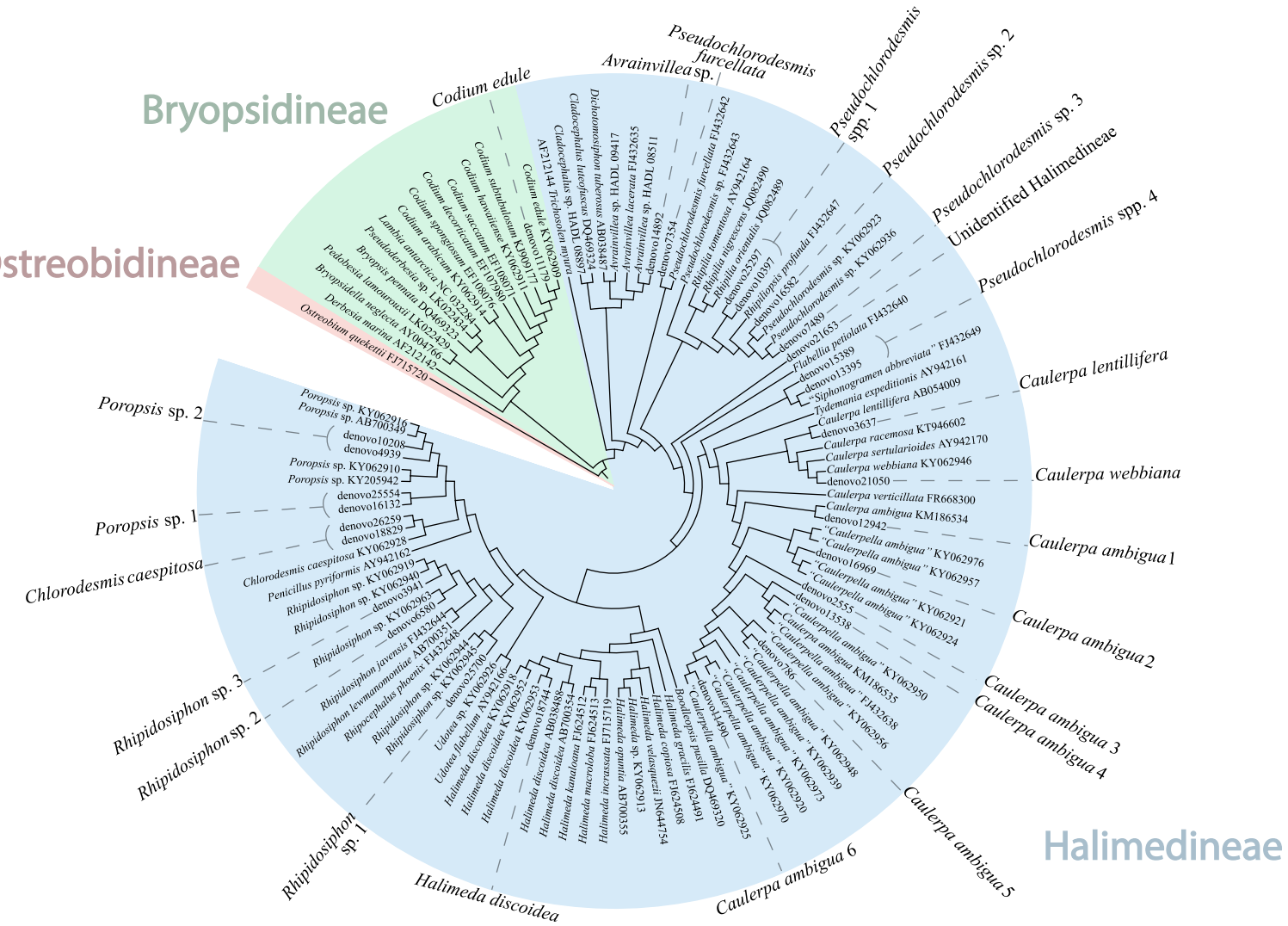


Figure 3.2. Maximum likelihood estimated phylogeny of the rbcL barcode sequence data. Branch lengths were ignored.

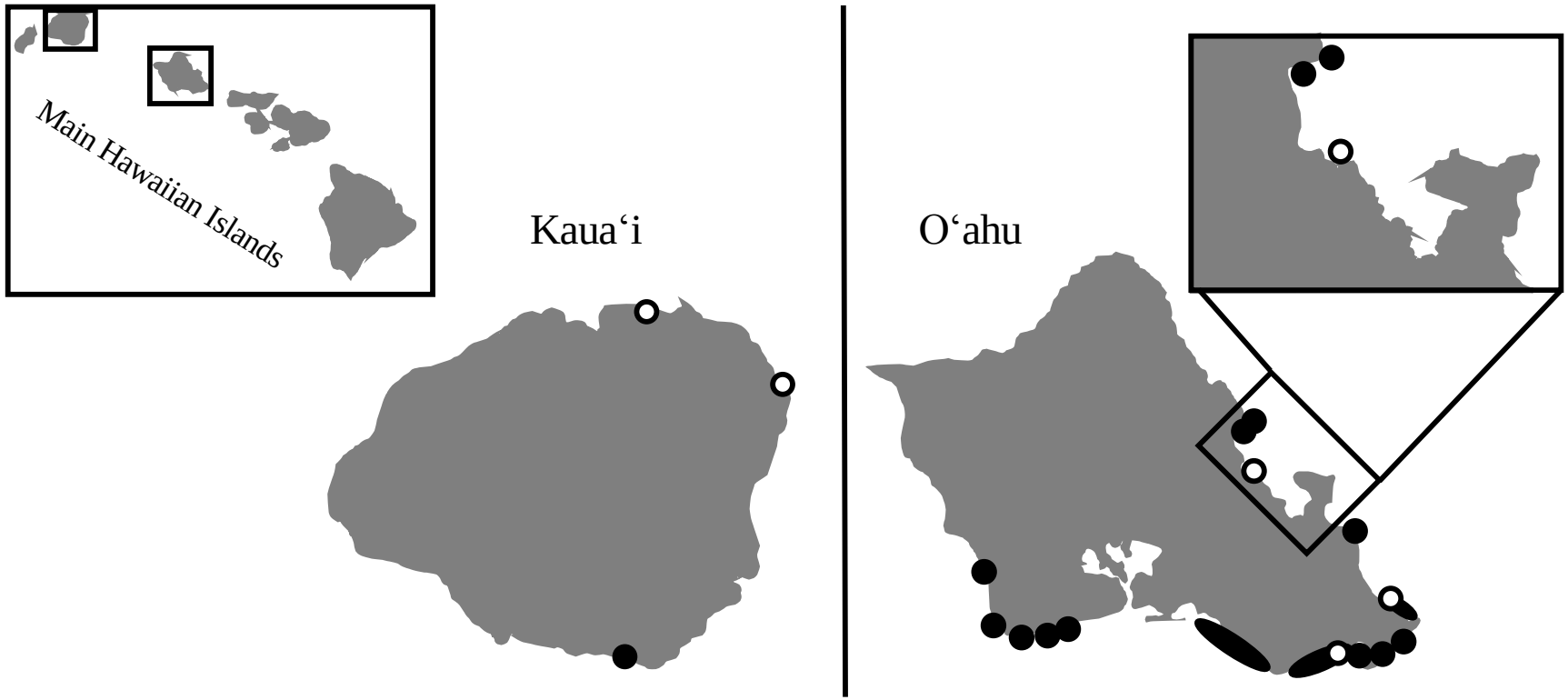
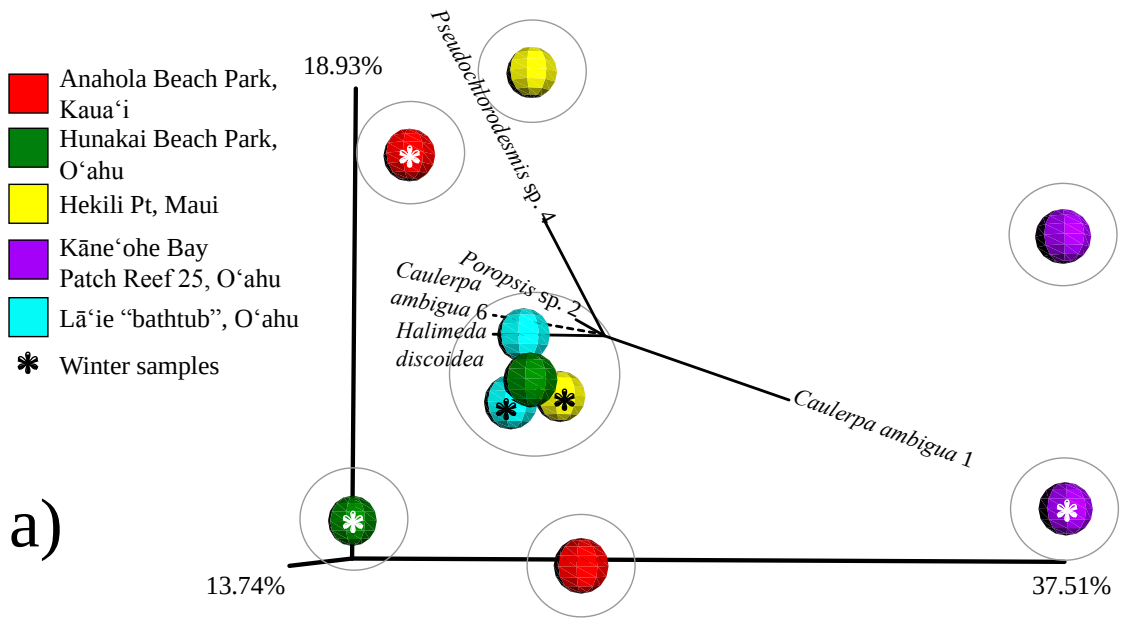
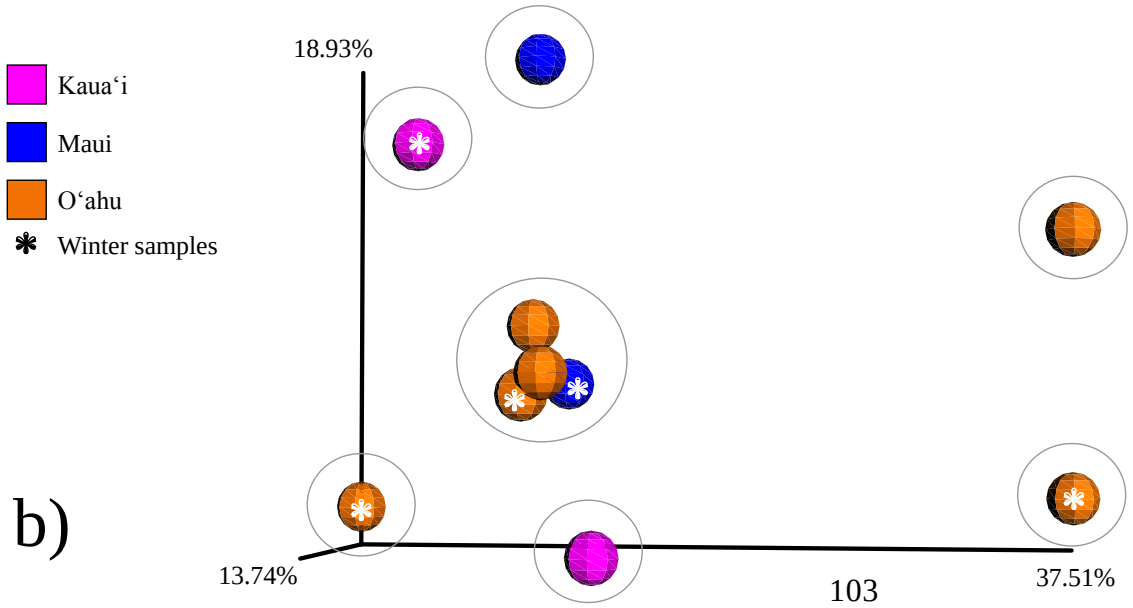


Figure 3.3. Map of previously recorded *Avrainvillea lacerata* populations (black) and newly recorded sites/populations (white) supported by *Plakobranchus* cf. *ianthobapsus* kleptoplast metabarcode data. Previous record data were provided by Brostoff et al. (1989), Smith et al. (2002), personal observations, or the Hawai‘i Division of Aquatic Resources.

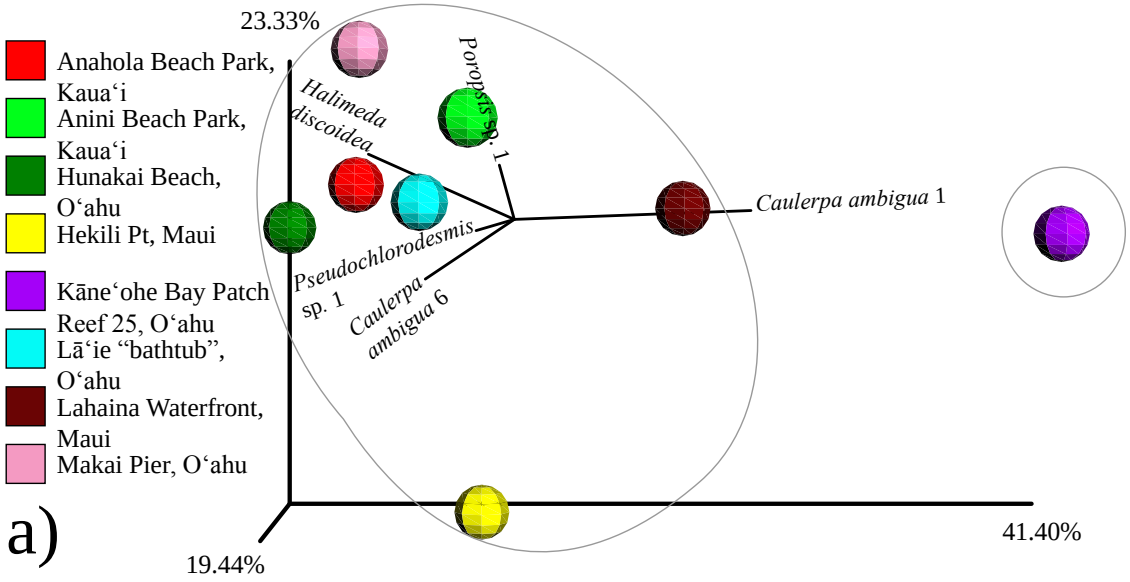

Anahola Beach Park, Kauaʻi
Hunakai Beach Park, Oʻahu
Hekili Pt, Maui
Kāneʻohe Bay Patch Reef 25, Oʻahu
Lāʻie "bathtub", Oʻahu
Winter samples
18.93%
Pseudochlorodesmis sp. 4
Caulerpa ambigua 6
Porpsis sp. 2
Halimeda discoidea
Caulerpa ambigua 1
13.74%
37.51%

a)

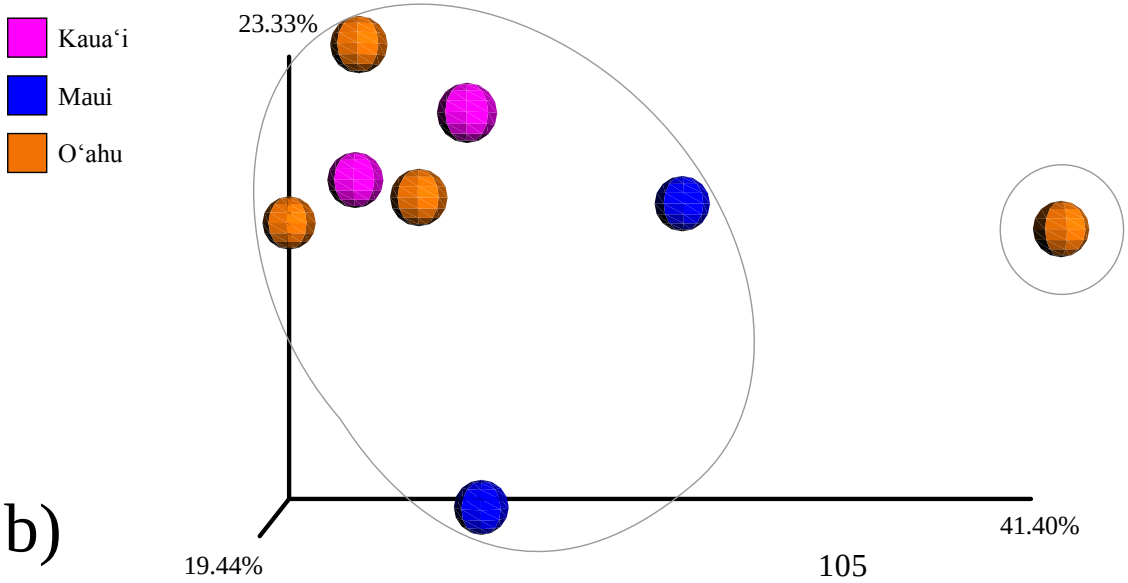

Kauaʻi
Maui
Oʻahu
Winter samples
18.93%
13.74%
37.51%

b)

Figure 3.4. Principal Coordinates Analysis (PCoA) of diversity dissimilarity among seasons within sites. Clustering is representative of similarity within sites, not among sites. A. Dissimilarity among seasons within sites. Sites are color-coded, winter season samples are identified with an asterisk. Effects of algal taxa driving the dissimilarity patterns are indicated by the branch length of the overlaid byplot. B. Dissimilarity among seasons within islands. Islands are color-coded, winter season samples are identified with an asterisk.


Anahola Beach Park, Kaua‘i
Anini Beach Park, Kaua‘i
Hunakai Beach, O‘ahu
Hekili Pt, Maui
Kāne‘ohe Bay Patch Reef 25, O‘ahu
Lā‘ie “bathtub”, O‘ahu
Lahaina Waterfront, Maui
Makai Pier, O‘ahu
23.33%
19.44%
41.40%
Halimeda discoidea
Porolithon sp. 1
Caulerpa ambigua 1
Pseudochlorodesmis sp. 1
Caulerpa ambigua 6

a)


Kaua‘i
Maui
O‘ahu
23.33%
19.44%
41.40%

b)

Figure 3.5. Principal Coordinates Analysis (PCoA) of diversity dissimilarity among sites with combined seasonal data. Clustering is representative of similarity based on Interquartile Range (IQR) ellipses produced by jackknifed beta diversity analyses. A. Dissimilarity among sites. Sites are color-coded, winter season samples are identified with an asterisk. Effects of algal taxa driving the dissimilarity patterns are indicated by the branch length of the overlaid byplot. B. Dissimilarity among islands. Islands are color-coded.

CHAPTER 4. Metabarcoding of epilithic algal communities confirms host selection specificity of *Plakobranchus* cf. *ianthobapsus'* (Sacoglossa, Gastropoda), increases known siphonous Hawaiian algal diversity, and expands the known range of *Avrainvillea lacerata* (Bryopsidales, Chlorophyta) in the Main Hawaiian Islands

Rachael M. Wade & Alison R. Sherwood

4.1 Abstract

Plakobranchus cf. *ianthobapsus* is a sacoglossan sea slug known for its generalist use of siphonous algal hosts and long-term retention of photosynthetically active plastids. While it is accepted that *P.* cf. *ianthobapsus* is a generalist herbivore with a species-rich list of algae from which it sequesters chloroplasts, without a complete taxonomic inventory of the siphonous algal community it is difficult to know what the proportion of this diversity is sequestered. *P.* cf. *ianthobapsus*' kleptoplast diversity data has provided crucial herbivory and record data for the highly invasive alga *Avrainvillea lacerata* (previously identified as *A. amadelpha*) in the Main Hawaiian Islands (MHI), these records would be best substantiated by direct algal data rather than sequestered plastid signal. To address the need for algal community diversity assessment and further definition of the current range of *A. lacerata* in Hawai'i, a metabarcoding approach was applied to epilithic algal communities at 19 sites, including those previously sampled for *P.* cf. *ianthobapsus* kleptoplast diversity studies, across the MHI during both summer and winter seasons. High throughput sequencing of an *rbc*L amplicon targeting siphonous green algae revealed 98 OTUs across all sites, demonstrating cryptic algal diversity in the bryopsidalean suborders Ostreobineae and Halimedineae and suggested the presence of undescribed species. Additionally, this survey of siphonous algal diversity revealed that *P.* cf. *ianthobapsus* only used 23 spp. (~23%) of species available in the siphonous green algal epilithic community, suggesting that *P.* cf. *ianthobapsus* is more specialized in its host selection than previously proposed. These data also show that *A. lacerata* has spread to three sites on the western shores of Maui – the first documented populations of the invasive alga outside of the islands of Kaua'i or O'ahu. This study demonstrates the utility of metabarcoding to not only increase our understanding of

algal diversity, but to also investigate herbivore ecology and invasive species range expansions.

Keywords: *Avrainvillea amadelpha*, cryptic diversity, environmental DNA, herbivore ecology, invasive species detection, *rbc*L

4.2 Introduction

Photosynthetic animals, especially kleptoplastidic or "chloroplast stealing" sea slugs have been featured in popular science and mainstream media repeatedly over the past decade for their intriguing ability to sequester and maintain photosynthetic chloroplasts from their algal hosts (see Moskowitz 2010; Jabr 2013; Fang 2015; Main 2018). *Plakobranchus* cf. *ianthobapsus* (Gould) is one such sea slug that is perhaps best known for its generalist host use of siphonous green algae (up to 23 different species; Maeda et al. 2012; Christa et al. 2013; Wade and Sherwood 2017, 2018) and long retention of photosynthetically active plastids (up to 11 months; Evertsen et al. 2007). While modern high throughput sequencing of kleptoplast amplicons (i.e. metabarcoding) has resulted in a more robust understanding *P.* cf. *ianthobapsus*' host use (Wade and Sherwood 2018), the degree of specificity of this host use is poorly defined without a clear examination of what the algal community has to offer,. Thus, the limits, and therefore ecological significance, of this slug's herbivory is still poorly understood. Furthermore, *P.* cf. *ianthobapsus* has been shown to preferentially sequester chloroplasts from diminutive (<2 cm at maturity), cryptic siphonous green algae (Wade and Sherwood 2017), making it difficult to catalog their diversity using traditional sampling and Sanger sequencing techniques. In this study, we address these shortfalls by applying a metabarcoding technique to epilithic algal communities across the Main Hawaiian Islands (MHI) to catalog siphonous algal diversity. This approach also allows a fine scale assessment

of the current distribution and range expansion of the highly invasive *Avrainvillea lacerata* (previously identified to as *A. amadelpha*) in Hawai‘i to confirm and complement the new records of the alga presented by Wade & Sherwood (2018). *A. lacerata* is of considerable concern in the MHI due to its rapid spread around the island of O‘ahu since its discovery in 1981 (Brostoff, 1989), persistence in both shallow and deep environments (Peyton, 2009; Spalding, 2012), and ability to colonize hard and soft substrate by altering the benthose (Cox et al., 2017; Foster et al., 2018), and therefore potentially threatening native, benthic flora and fauna.

4.3 Methods

4.3.1 Sample Collection

Twenty pieces of live rock (i.e. rubble or rock chips with epilithic algae) were extracted using hammers and chisels via snorkeling from 19 sites across the MHI in Summer 2017 (May 25-July 23) and Winter 2018 (January 27-March 19); a fifth site on Kaua‘I, Kekaha Beach Park, was targeted, but was not sampled in winter or summer due to high surf and strong currents on dates visited. These sites were selected based on their use for *Plakobranchus* cf. *ianthobapsus* kleptoplast diversity studies (Wade & Sherwood 2017, 2018), accessibility, and ability to provide an even distribution along each island’s coastline. These sites also offer a diversity of geomorphological structure (e.g. available substrate) and biological cover (BAE Systems 2007; Table 4.1), and thus increased the probability of maximum algal diversity and coverage of *Avrainvillea* presence/absence data for the archipelago.

4.3.2 Illumina Library Preparation and Sequencing

Using the methods of Sauvage et al. (2016), algal communities were shaved from the rocks using a Dremel 2000 rotary tool with Flex Shaft Attachment fitted with a sterile 1.6mm (1/16”) bit. Bit and attachment hardware were sterilized between sites using a 10% bleach

solution for at least 15 minutes. All samples were pooled by site and season (n=38) and mixed thoroughly before a 250 mg subsample of the pooled community was frozen and subsequently extracted using an OMEGA biotek E.Z.N.A.® Soil DNA Kit (Norcross, GA, U.S.A). Negative controls were also included during each extraction period (n=2). DNA extracts were then prepared for multiplexed high throughput sequencing using a modified protocol after "16S Metagenomic Sequencing Library Preparation: preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system" protocol (Illumina 2013). An *rbc*L amplicon (376 bp), an internal region of the Pierce et al. (2006) barcode (562 bp), was amplified using the forward primer therein (rbcLF) and a newly designed reverse primer (rbcL400R). *rbc*L is a prescribed DNA barcode for siphonous green algal species delimitation (Leliaert et al., 2014) and has been used in previous metabarcoding study (Wade and Sherwood, 2018). *rbc*L was first amplified using primers with Illumina overhang adapters. Indices and Illumina sequencing adapters were annealed to successful amplicons in a secondary amplification step. Libraries were then size selected and purified using Omega Mag-Bind® TotalPure NGS beads (Norcross, GA, U.S.A). Lastly, libraries were quantified using a Life Technologies Qubit™ 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), normalized, and pooled before submission for sequencing on an Illumina MiSeq platform for separate, partial 600 cycle runs at the University of Hawai'i at Mānoa Advanced Studies in Genomics, Proteomics and Bioinformatics facility.

4.3.3 Data analyses

All scripts are included in Supplementary Materials. Loss of data with each data processing step is summarized in Table 4.2. Read quality was first assessed using FastQC (Andrews 2010), then filtered for quality and merged using PEAR (Zhang et al., 2014). Sequences were prepared for analysis using the Qiime 1 pipeline (Caporaso et al., 2010). Once an OTU table

was generated, singletons and sequences from extraction and amplification controls (n=4) were used to filter the data. Abundance thresholds of 0.1% and a minimum of 2 reads were used to consider an OTU a true positive in control samples; OTUs below these thresholds were not considered true contaminants. A more relaxed abundance threshold of 0.005% was applied to OTUs found in study samples to reduce phylogenetic noise; OTUs above this threshold were considered true members of the sampled community (Bokulich et al., 2013). These abundance thresholds were applied particularly to provide confidence in the assessment of the presence/absence of *Avrainvillea lacerata*. Additional phylogenetic assessment of OTUs returning "None" for taxonomic assignment was conducted; because of low genetic divergence of the amplicon in some lineages, particularly in the terminal branches of the Udoteaceae (e.g. *Poropsis* and *Rhipidosiphon* lineages), traditional alignment-based taxonomic assignment methods resulted in multiple identifications with the same E value and therefore cannot provide a single confident identification. Thus, further assessment of these OTUs is necessary. Representative siphonous green algal OTU sequences that passed all filtering were extracted and used for further phylogenetic analysis using Geneious (Kearse et al., 2012): sequences were aligned with published reference sequences using MUSCLE (Edgar, 2004) and exported for phylogenetic reconstruction using RAxML (v. 8.2.10, Stamatakis 2014) and MrBayes (v. 3.2.6, Ronquist et al. 2012) via the CIPRES science gateway (Miller et al., 2010).

4.4 Results

4.4.1 Siphonous algal diversity

The *rbc*L phylogenetic reconstruction supported 98 siphonous green algal OTUs from the orders Bryopsidales and Dasycladales. Only two OTUs corresponded to dasycladalean taxa – *Bornetella sphaerica* and *Neomeris* sp. The remaining OTUs represent species

spanning the order Bryopsidales with representatives throughout all three bryopsidalean suborders.

4.4.2 Cryptic species and putative new lineages

Several diminutive lineages were found to harbor cryptic putative new species, particularly within the bryopsidalean genera *Ostreobium*, *Bryopsis*, *Derbesia*, *Pedobesia* and *Pseudoderbesia* (Fig. 4.1). In the MHI, these genera are fairly depauperate (records include *O. queketti*, *Bryopsis hypnoides*, *B. pennata*, and *B. plumosa* var. *secunda*, *Derbesia fastigiata* and *D. tenuissima*) or are currently absent from the records (*Pedobesia, Pseudoderbesia*, members of the Pseudostreobineae) (Guiry and Guiry, 2019). Within most of these genera several OTUs were recovered (*Ostreobium* n=18, *Bryopsis* n=6, *Derbesia* n=1, *Pedobesia* n=3, *Pseudoderbesia* n=1, Pseudostreobineae, n=9; Fig. 4.1).

Additionally, there are several putative new lineages (Fig. 4.1): a clade of 9 OTUs sister to but distinct from *Derbesia* (8.86-14.18% divergent from *Derbesia*), a clade of 2 OTUs diverging early in the Bryopsidaceae (9.11-18.10% divergent from other members of the Bryopsidaceae), a clade of 4 OTUs sister to but distinct from *Bryopsis* (6.58-12.15% divergent from *Bryopsis*).

4.4.3 *Avrainvillea lacerata* distribution & and range expansion

Avrainvillea lacerata was recovered from all sites sampled around the island of Oʻahu at both the 0.1% and 0.005% thresholds. These records reflect confirmation of previous records at four of the sites and a new record at the Lāʻie site, which is farther north than the other sites and any previous records (Fig. 4.2). "*A. amadelpha*" was also recovered from three of the sites on Maui – Hekili Pt (Winter), Lahaina Waterfront (Winter), and Wailea Beach Park (Winter); the alga was only present at Wailea Beach Park at the 0.005% threshold while others met the 0.1% minimum. The recent records at Anini and Anahola Beach Parks on

Kaua‘i from Wade & Sherwood (2018) were not confirmed in this study; *A. lacerata* was recovered from the Anini Beach Park (Winter) samples but was below the 0.005% threshold (n=1 read). This invasive alga was not recovered from any other sites on Kaua‘i, including Prince Kuhio Beach site (Smith et al. 2002), or any sites on Hawai‘i above or below either thresholds. No sequences matching *A. erecta* (Wade et al. 2018) were recovered in this study.

4.5 Discussion

4.5.1 Cryptic algal diversity

This study is one of several to demonstrate the power of metabarcoding to explore cryptic algal diversity (e.g. Marcelino and Verbruggen 2016; Sauvage et al. 2016; Groendahl et al. 2017; Wade and Sherwood 2018). Of the cryptic species OTUs recovered, many are not identical to published sequences and are therefore potential new species (*Ostreobium* n=15, *Bryopsis* n=6, *Pedobesia* n=2, *Pseudoderbesia* n=1). Given the challenge of morphological species identifications of many of these taxa (e.g. Kooistra 2002; Verbruggen et al. 2009; Sauvage et al. 2016), these putative new phylogenetic species are of considerable value in our understanding of siphonous green algal diversity. Further, this study and others using similar techniques have the opportunity to contribute to algal diversity estimates. Most recent estimates place algal diversity in the ballpark of 72,500 (Guiry 2012) to 170,000 species (De Clerck et al., 2013). If the results of this study are any indication of known overall algal diversity (i.e, that up to 25% of the species recovered are likely new to science), then these estimates are likely quite low.

4.5.2 *Plakobranchus* cf. *ianthobapsus* host use limitations

Representation of total siphonous green algal diversity in the MHI allows direct comparison of this diversity with *P.* cf. *ianthobapsus* kleptoplast diversity recovered in previous studies (Wade and Sherwood 2017, 2018). Discussion of *P.* cf. *ianthobapsus* as an herbivore most

often results in its categorization as a generalistic specialist: while it has been previously reported to sequester chloroplasts from up to 23 species of algae (Wade and Sherwood 2018), nearly all of those species are siphonous green algae from the bryopsidalean suborder Halimedineae. However, it is difficult to discuss host limitations and herbivore ecology without a thorough understanding of what is available in the community. The results of the present study suggest that despite having up to 98 siphonous green algal species to choose from, *P.* cf. *ianthobapsus* only acquires chloroplasts from ~23% of them (up to 23 spp.; Fig. 4.3). These 23 species are almost entirely from the suborder Halimedineae; only *Codium edule* from the Bryopsidineae is utilized as a kleptoplast source, and this taxon is considered ecologically insignificant due to how rarely it is used (Wade and Sherwood 2018). This phylogenetically delineated host use is supported by significant differences among these suborders in terms of their physiology, chemistry, and biology. The Bryopsidineae have mannan (sporophytic stages of Bryopsidaceae; Codiaceae) or xylan and cellulose (gametophytic stages of Bryopsidaceae) as their principal cell wall components, while the Halimidineae are composed primarily of xylan (Hillis-Colinvaux, 1984), which could be driving limitations of tooth morphology and therefore *P.* cf. *ianthobapsus*' ability to pierce the cell wall (Marín and Ros, 2004). The Ostreobineae and most genera within the Bryopsidineae are uniaxial (Codiaceae are multiaxial), while all but the Caulerpaceae in the Halimedineae taxa are multiaxial (Hillis-Colinvaux, 1984), although it is important to note that all siphonous green taxa are likely uniaxial as juveniles. Lastly, the Bryopsidineae are homoplastic while the Halimedineae are heteroplastic (Graham et al., 2009; Hillis-Colinvaux, 1984), which could play a role in selection of algal hosts as both kleptoplast and nutrition sources. Overall, these differences in algal biology may be key drivers of host selection and

limitation and provide an explanation of why *P.* cf. *ianthobapsus* appears to be a much more specialized herbivore than perhaps previously estimated or portrayed.

4.5.3 *Avrainvillea lacerata*'s continued invasion in the MHI

This study provides a fine-scale sampling of epilithic algal communities as part of an effort to confirm *A. lacerata*'s known range and to provide data on any expansions, particularly outside the island of O'ahu. Environmental data (eDNA) is becoming an increasingly popular invasive species detection tool (e.g. Aibin et al., 2017; Jerde et al., 2011; Rees et al., 2014; Takahara et al., 2013). The data presented here suggest that *A. lacerata* dramatically expanded beyond both of the islands of Kaua'i and O'ahu to the eastern lying island of Maui. These new populations are not completely surprising given the results of recent modeling efforts to predict areas at high risk of invasion by the alga, which suggested the western shores of Maui as highly suitable environment for the alga to invade and persist (Veazey et al., in revision). Additionally, repeated traditional surveying from 2014-2018 (R. Wade, unpublished data) and metabarcoding of the epilithic communities of Prince Kuhio Beach, Kaua'i - the first reported sighting of *A. lacerata* outside O'ahu (Smith et al., 2002) – suggest that this population is no longer persisting and may be absent at the site entirely. While the data presented here do not necessarily support the previous report of two new populations on the northeastern shores of Kaua'i (Wade & Sherwood, 2018), continued efforts should be made to monitor these sites. In rare cases, it appears that *Plakobranchus* cf. *ianthobapsus* may be a better sampling tool than the methods employed here (see gold highlighted taxa in Fig. 4.3). Thus, *A. lacerata* at these sites may have been at low enough abundance and/or overwhelmed by the biomass of other taxa in the epilithic community in the summer of 2017 to elude detection. Particularly because these sites are also predicted to be at high risk for invasion (Veazey et al., in revision),

it will be important to revisit these sites and continue to use metabarcoding as a detection method for this highly invasive species.

4.6 Conclusions

Metabarcoding of algal communities provides a low cost, fine scale method of collecting an incredible amount of data with diverse applications. Particularly when traditional biodiversity collection and identification methods fall short in their ability to fully catalog species, metabarcoding can be an approachable option. Furthermore, when an invasive species is continuing to spread and first appears in communities in dimunitive, cryptic form, metabarcoding provides a DNA-based detection tool that can undoubtedly outperform visual detection methods.

4.7 Permitting Information

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4.11 Tables

Table 4.1. Collection site characterization of abiotic and biotic data.

Island	Site	Latitude	Longitude	Winter Date Sampled	Summer Date Sampled	Zone	Geomorphological Structure	Biological Cover
Hawai ‘i	Onekahakaha Beach Park	19.738325	-155.03868	2-Mar-2018	25-May-2017	Reef Flat	Rock/Boulder	Turf 10-<50%
Hawai ‘i	Richardson Beach Park	19.734125	-155.01607	2-Mar-2018	25-May-2017	Reef Flat	Rock/Boulder	Macroalgae 10-<50%
Hawai ‘i	Hapuna Beach Park	19.99452	-155.82696	1-Mar-2018	-	Bank/ Shelf	Sand	Uncolonized 90-100%, Macroalga

								e 50-<90%
Hawai‘i	Anaeho‘omalu Beach	19.91395	-155.888275	1-Mar-2018	24-May-2017	Bank/Shelf	Sand, Rock & Boulder	Turf 50-90%, Uncolonized 90-100%
Hawai‘i	Kahalu‘u Beach Park	19.57919	-155.96805	1-Mar-2018	24-May-2017	Bank/Shelf	Rock/Boulder	Turf 50-<90%
Kaua‘i	Anahola Beach Park	22.14645	-159.29850	27-Jan-2018	24-Jun-2017	Reef Flat	Pavement	Macroalgae 10-<50%
Kaua‘i	Anini Beach Park	22.22421 6	-159.44646	27-Jan-2018	24-Jun-2017	Reef Flat	Pavement	Turf 50-<90%

Kaua‘i	Lydgate State Park	22.04145	-159.33429	27-Jan-2018	24-Jun-2017	Reef Flat	Sand	Uncolonized 90-100%
Kaua‘i	Prince Kuhio Beach Park	21.88089	-159.4737	28-Jan-2018	25-Jun-2017	Bank/Shelf	Rock/ Boulder	Turf 50-<90%
Maui	Hekili Pt	20.809093	-156.62288	18-Mar-2018	8-Jul-2017	Reef Flat	Pavement	Macroalgae 10-<50%
Maui	Lahaina Waterfront	20.865004	-156.67394	18-Mar-2018	8-Jul-2017	Reef Flat	Pavement, Sand	Macroalgae 10-<50%, Uncolonized 90-100%

Maui	D.T. Fleming Beach Park	21.00564	-156.65169	-	8-Jul-2017	Bank/Shelf	Sand, Rock/Boulder	Uncolonized 90-100%, Turf 50-<90%
Maui	Ho‘okipa Beach Park	20.93469	-156.35635	17-Mar-2018	9-Jul-2017	Reef Flat	Pavement	Turf 50-<90%
Maui	Wailea Beach Park	20.68145	-156.44429	17-Mar-2018	9-Jul-2017	Bank/Shelf	Sand, Scattered Coral/Rock	Uncolonized 90-100%, Turf 10-<50%
O‘ahu	Hunakai Beach Park	21.262764	-157.783565	19-Mar-2018	22-Jul-2017	Reef Flat	Pavement	Macroalgae 10-<50%

O‘ahu	Kāne‘ohe Bay Patch Reef 25	21.460839	-157.823121	12-Mar-2018	17-Jun-2017	Fore Reef/Reef Flat	Individual Patch Reef	Coral 10-50%
O‘ahu	Lā‘ie "Bath tub"	21.635759	-157.918672	14-Mar-2018	23-Jul-2017	Reef Flat	Sand	Uncolonized 90-100%
O‘ahu	Makai Pier	21.262764	-157.783565	14-Mar-2018	22-Jul-2017	Reef Flat	Pavement	Macroalgae 10-<50%
O‘ahu	Maunalua Bay via Paiko Rd	21.281105	-157.728191	18-Mar-2018	22-Jul-2017	Reef Flat	Pavement	Macroalgae 10-100%

Table 4.2. DNA sequence data remaining after each step of quality control filtering and data analysis using the QIIME pipeline and phylogenetic species assignment of sequence data. "None" refers to samples that the taxonomic assignment step returned multiple identifications with the same confidence score, and thus a confident identification could not be made.

		% Raw Data	# Reads	#OTUs	% OTUs
Pipeline Step	Raw Sequences	100	4,201,392		
	Read Merging w/ QC filtering	92.76	3,881,704		
	Chimera removal	91.35	3,822,319		
	Denoising (removal of singletons)	90.63	3,792,104		
	Decontamination (removal of control sample & non-algal OTUs)	89.72	3,769,423	8,034	100
Taxonomic breakdown	No blast hit	9.20	386,619	6647	82.74
	"None"	5.62	236,075	635	7.90
	Non-siphonous algae (e.g. cyanobacteria, green algae)	28.34	1,190,671	574	7.1
	Siphonous green algae	50.28	2,342,677	178	2.21

Siphonous green algae with additional filtering	Siphonous green algae + re-evaluated "None"	61.17	2,570,376	205	2.55
	All siphonous green algae ≥ .005% reads	55.70	2,340,192	103	1.28

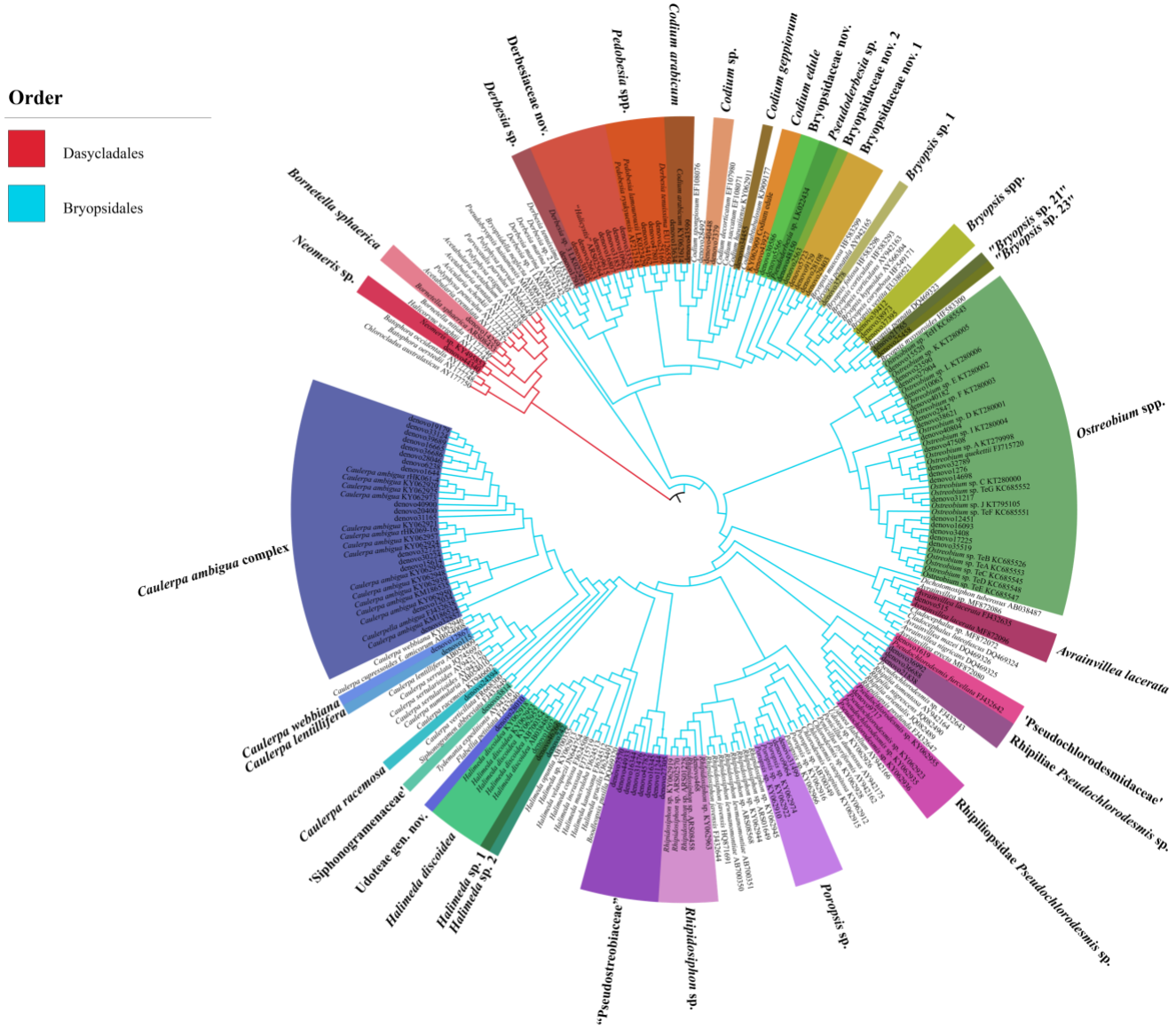
Order
Dasycladales
Bryopsidales
Derbesia sp.
Derbesiaceae nov.
Pedobesia spp.
Codium arabicum
Codium sp.
Codium geppiorum
Codium edule
Bryopsidaceae nov.
Pseudodelesia sp.
Bryopsidaceae nov. 2
Bryopsidaceae nov. 1
Bryopsis sp. 1
Bryopsis spp.
"Bryopsis sp. 21"
"Bryopsis sp. 23"
Ostreobium spp.
Avrainvillea lacerata
'Pseudochlorodesmidaceae'
Rhipiliae Pseudochlorodesmis sp.
Rhipiliopsidae Pseudochlorodesmis sp.
Pseudostreobiaceae"
Rhipidosiphon sp.
Poropsis sp.
Halimeda sp. 1
Halimeda sp. 2
Halimeda discoidea
Udoteae gen. nov.
'Siphonogramenaceae'
Caulerpa racemosa
Caulerpa webbiana
Caulerpa lentillifera
Caulerpa ambigua complex
Neomeris sp.
Bornetella sphaerica

Figure 4.1. Maximum likelihood reconstruction of siphonous algal community *rbc*L metabarcode data with recovered taxa highlighted. Branch color corresponds to the provided legend delineating taxonomic Order.

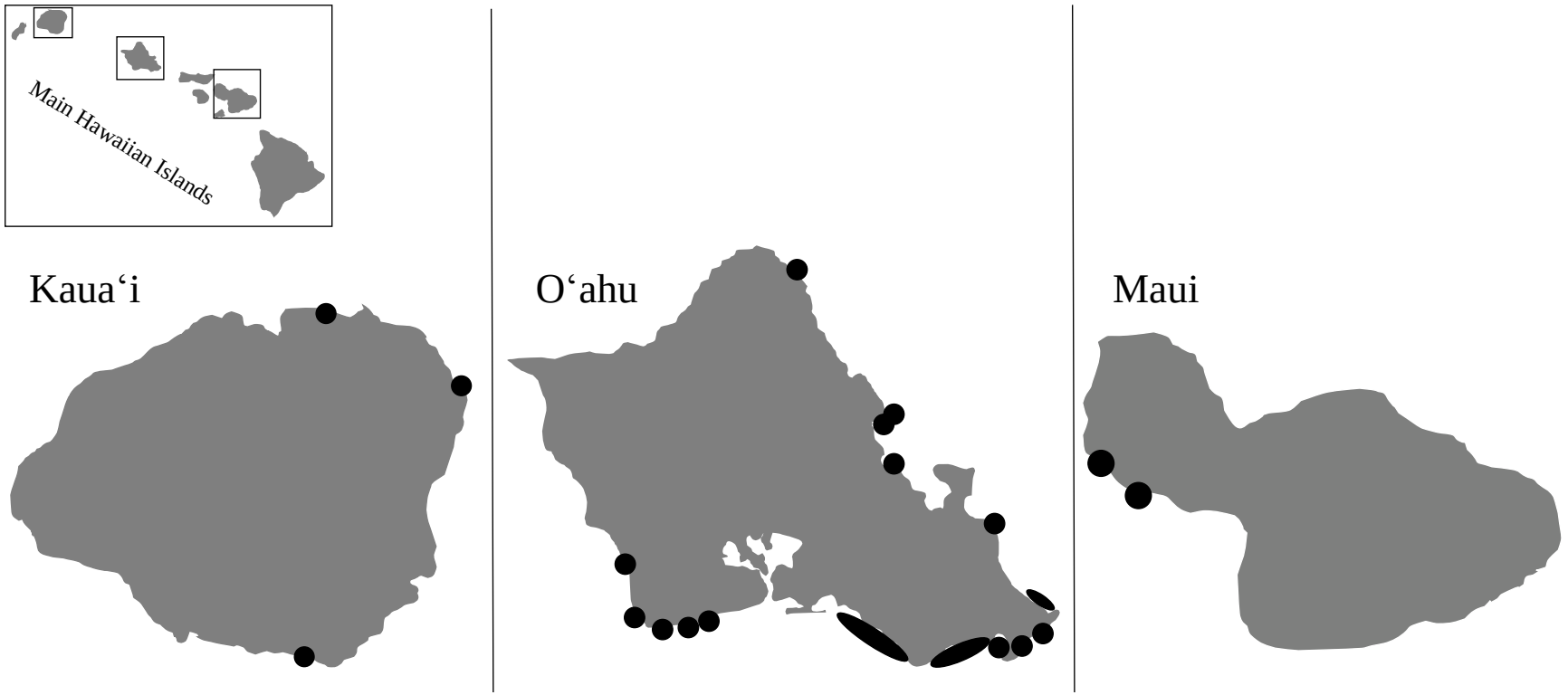

Fig. 4.2. *Avrainvillea lacerata* distribution in the Main Hawaiian Islands using published reports and data and the results of this study.

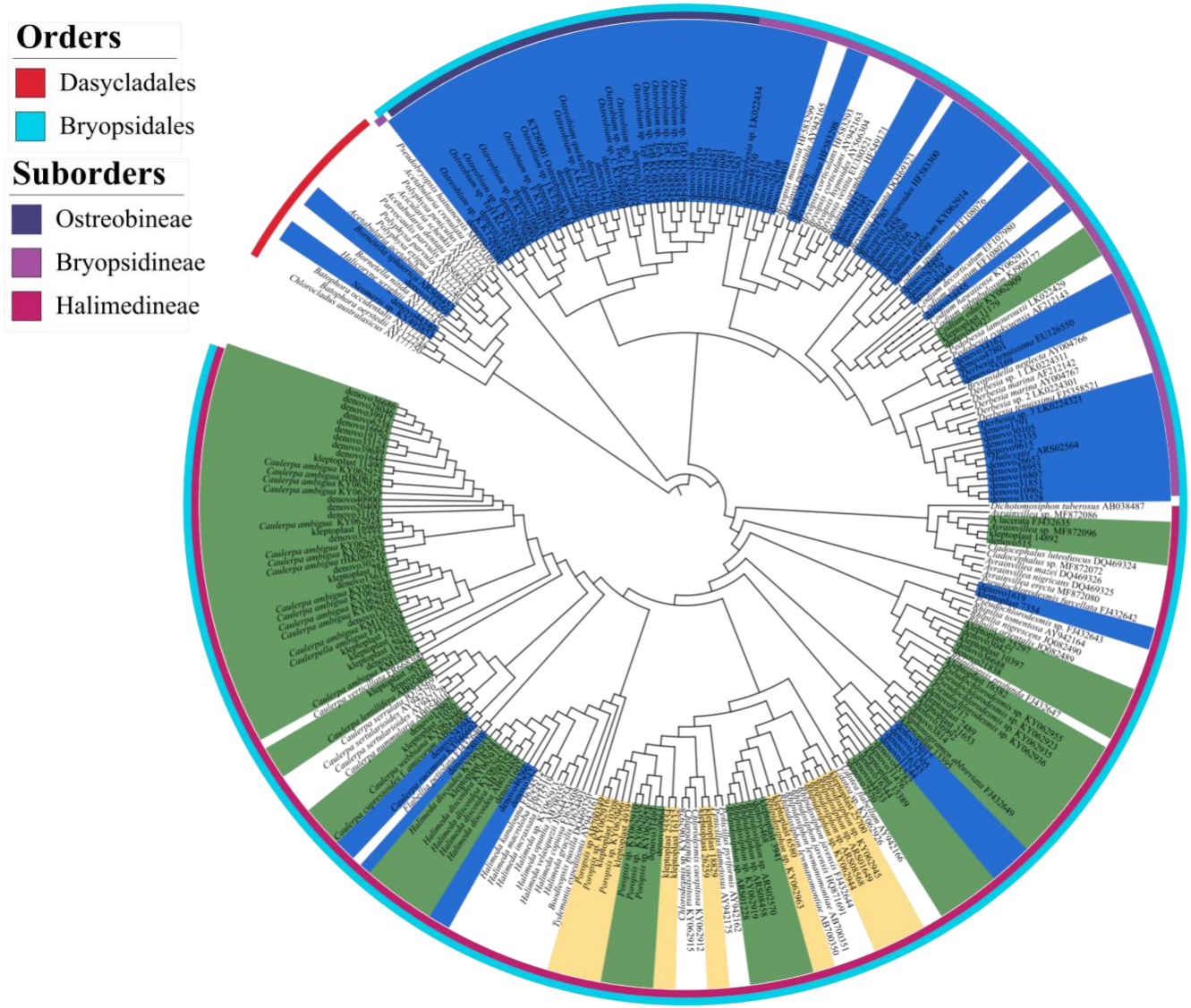
Orders
Dasycladales
Bryopsidales
Suborders
Ostreobineae
Bryopsidineae
Halimedineae

Fig 4.3. Maximum likelihood reconstruction of rbcL metabarcode data comparing what was recovered from the epilithic algal communities and the kleptoplast diversity reported in Wade & Sherwood (2018).

CHAPTER 5. Molecular systematics of the siphonous green alga *Avrainvillea* (Dichotomosiphonaceae, Bryopsidales) with an emphasis on invasive species identification

Rachael M. Wade[a], Thomas S. Sauvage, and Alison R. Sherwood[a]

5.1 Abstract

Avrainvillea is a siphonous green alga found throughout the Caribbean, Indo-Pacific, and has more recently invaded the southern, subtropical portions of the Mediterranean Sea. Systematic treatments of the genus are few in number and have exclusively relied on morphological characters. This is particularly problematic as many of the currently recognized species were described in the late 19th century and their descriptions often include only gross morphological features. Furthermore, like many other siphonous green algal genera, *Avrainvillea* exhibits morphological plasticity. *Avrainvillea* is also of particular interest because of three documented expansions outside its recorded native range: "*A. amadelpha*" and *A. erecta* in the Main Hawaiian Islands and *A. amadelpha* in the Mediterranean Sea. Using the most recent taxonomic scheme developed for the genus, the identifications of these species were determined using morphological characters alone. In this study we use two chloroplast gene regions to reconstruct a molecular phylogeny, demonstrating that morphological characters are not reliable for *Avrainvillea* species delimitation and that specimens previously identified by leading phycological taxonomists exhibit rampant polyphyly. Neither the Hawai'i nor Mediterranean invader are here identified as *A. amadelpha* – the Hawai'i species grouped with the *A. lacerata* isotype specimen, while the identification of the Mediterranean species remained elusive. *A. obscura* specimens from Guam, the type locality, further supported the identification of the recently recorded *A. erecta* in the Main Hawaiian Islands as a correct identification. Given the issues associated with morphological identification if *Avrainvillea*, further efforts to resolve the phylogeny of the genus should incorporate DNA sequence data of type specimens, to the degree possible.

Keywords: *Avrainvillea amadelpha*, *Avrainvillea lacerata*, Hawai‘i, Mediterranean Sea, *rbc*L, *tuf*A, type specimens

5.2 Introduction

Avrainvillea is a siphonous green alga found throughout the Caribbean and Indo-Pacific, and has more recently invaded the southern, subtropical portion of the Mediterranean Sea (Verlaque et al., 2017). *Avrainvillea* currently comprises 37 species with limited distribution between the Caribbean and Pacific (i.e. *A. longicaulis*, *A. nigricans, A. mazei* are recorded in both ocean basins; Guiry and Guiry, G.M., 2019). The systematics of this genus have long been fraught with complication beginning with two independent descriptions of the same taxon: *Avrainvillea* Decaisne (Decaisne, 1842) and *Fradelia* Chauvin (Chauvin, 1842), which were soon after synonymized (Murray and Boodle, 1889). Within 20 years, *Chloroplegma* Zanardini (Zanardini, 1857) was described, but is now also considered synonymous with *Avrainvillea* (Murray and Boodle, 1889). *Avrainvillea*'s family-level placement has also been uncertain with its original placement in the Udoteaceae (Murray and Boodle, 1889), but later transferred to the Dichotomosiphonaceae by Curtis et al., (2008), which has been supported by additional phylogenetic and phylogenomic analyses (Cremen et al., 2019; Verbruggen et al., 2009).

Systematic treatments of the genus *Avrainvillea* are few in number (Littler and Littler, 1992; Olsen-Stojkovich, 1985) and have exclusively relied on morphological characters. More specifically, these treatments have distinguished groups based on gross morphological features such as stipe presence/absence, blade shape, and holdfast morphology, while species designations were primarily distinguished by microscopic features such as siphon morphology, size, and dichotomy presence/absence and constriction. This separation of macro- and micro-scopic characters is particularly problematic because many of the currently

recognized species were described in the late 19th century or early 20th century, and their descriptions often do not often include detailed microscopic features. Furthermore, like many other siphonous green algal genera, *Avrainvillea* is phenotypically plastic in its morphology (Brostoff, 1989; Olsen-Stojkovich, 1985); clearly so at the macroscopic scale, but also to some degree at the microscopic level. Thus, there is a real need for molecular assessment of this genus across its range to better understand and identify species limits and phylogenetic relationships.

A robust molecular phylogeny is also of interest because of the pervasiveness of invasive *Avrainvillea* spp. and the need for their rapid and accurate identification. The first documented invasion of an *Avrainvillea* sp. was discovered off the west coast of O‘ahu in the Main Hawaiian Islands (MHI) in 1981. Brostoff (1989) identified this alga as *A. amadelpha* (Montagne) A. Gepp & E.S. Gepp, but noted that the alga also morphologically resembled *A. hollenbergii* Trono, *A. lacerata* J. Agardh, and *A. riukiuensis* Yamada, most likely because of morphological plasticity. By 1985, “*A. amadelpha*” had infiltrated much of the shallow coastal habitat along O‘ahu’s south shore. In 2002, expansive meadows of “*A. amadelpha*” were discovered as deep as 90 m, also along the south shore of O‘ahu (Spalding, 2012). Today, “*A. amadelpha*” is a dominant member of both the shallow and mesophotic environments surrounding O‘ahu with recently documented invasions in the shallows of the neighboring islands of Kaua‘i (Smith et al., 2002; Wade and Sherwood, 2018) and Maui (Wade and Sherwood, in prep). In addition to rapid spread in the MHI, “*A. amadelpha*” is also of serious concern because of its ecological impact. Due to its ability to collect sediment using its robust, mat-like holdfast, and resulting modification of the benthos, “*A. amadelpha*” is able to colonize both hard and soft substrate (Foster et al., 2018; Peyton, 2009; Spalding, 2012).

Additionally, "*A. amadelpha*" is resistant to fluctuations in temperature and exposure, allowing it to become a dominant member of intertidal benches, excluding local algal species, and reducing overall diversity (Cox et al., 2017; Foster et al., 2018).

In 2012, the Sherwood Algal Biodiversity Lab at the University of Hawai‘i at Mānoa was contacted by Dr. Ali Ahmed El Fituri who had recently collected and documented an unknown *Avrainvillea* sp. off the coast of Tivoli, Libya. In 2014, an alga identified as *A. amadelpha* was discovered off the coast of Tunisia in the Mediterranean Sea less than 100 km from Dr. El Fituri's collection site (Verlaque et al., 2017). While thorough morphological examination, including reproductive features, was conducted, molecular data was not successfully produced by Verlaque et al., (2017). Thus, using the morphological features only, the authors identified the invader as *A. amadelpha*.

An alga identified as *Avrainvillea* cf. *erecta* has also recently invaded the MHI (Wade et al., 2018). Two populations were discovered off the south shore of O‘ahu in urbanized estuaries of Mālama Bay – one in Honolulu Harbor in 2014, another seaward of Ke‘ehi Lagoon in 2017. The identification was tentative as incorporation of basionym type specimens of *A. erecta* was not possible at the time of publication. Thus, additional work to clarify the identity of the non-native species is needed.

The goals of this study are to generate and analyze a molecular phylogeny of the siphonous green algal genus *Avrainvillea* in order to 1) evaluate previously published non-native and invasive species identifications in Hawai‘i (Brostoff, 1989; Wade et al., 2018) and the Mediterranean Sea (Verlaque et al., 2017), 2) evaluate the usefulness of morphological characters for species delimitation and previously described morphometric clusters (Olsen-Stojkovich, 1985), and 3) provide a robust DNA sequence-based framework for the rapid and

accurate identification of *Avrainvillea* spp. to allow timely evaluation of future invasions by members of this genus.

5.3 Methods

Specimens and/or DNA extracts representing up to *Avrainvillea* species from both the Caribbean and Indo-Pacific were freshly collected, donated by collaborators, or loaned from museum and/or herbaria collections (Table 5.1). Type specimens of *A. amadelpha* (holotype, PC42670), *A. clavatiramea* A. Gepp & E.S. Gepp (type, BM000515983) *A. gardineri* A. Gepp & E.S. Gepp (type, BM000515984), *Avrainvillea lacerata* (isotype, BM000515991), *A. ridleyi* A. Gepp & E.S. Gepp (isosyntype, BM000515995). Non-type specimens collected from type localities included *A. obscura* (C. Agardh) J. Agardh (BM001044690, GLS031, RT2019) and *A. riukiuensis* (BM000569543). For type specimen loans, destructive sampling was only permitted for molecular assessment, thus morphological assessment was not conducted on types. Because of this limitation and the known challenges of reliable and consistent morphological characters for this genus, this study focused primarily on molecular phylogenetic reconstruction of *Avrainvillea* to infer identity. All molecular work involving type specimens was conducted following the recommendations from Hughey and Gabrielson, (2012) – filtered tips were used for all extraction and amplification work, tubes were autoclaved and further disinfected using a UV decoupler, an extraction control was included during each round of DNA extraction and amplification, and extractions were conducted in a lab that does not undertake any phycological research. Genomic DNA was extracted using either an OMEGA E.Z.N.A Plant DNA Kit (OMEGA bio-tek, Norcross, GA U.SA.) or the protocol described by Hughey et al., (2001), the latter being prioritized for type specimens, older specimens (i.e. >25 years old) and/or specimens that failed to yield successful sequences when using the OMEGA kit. Portions of two chloroplast gene regions were targeted: the 5'

end of *rbc*L (ribulose-1,5-bisphosphate carboxylase/oxygenase – large subunit, 562 bp) and *tuf*A (elongation factor Tu, 714 bp) (Händeler et al., 2010; Wade and Sherwood, 2017), which are recommended as informative loci for siphonous green algal molecular systematics (Leliaert et al., 2014). For older and type specimens, modified protocols amplifying ~100-200 bp overlapping fragments within each loci were used to increase amplification and sequencing success (Table 5.2; Wade et al., 2018). When these methods failed for type specimens (i.e., *A. amadelpha*, *A. lacerata*, and *A. ridleyi*), genomic DNA was sent to myGenomics, LLC (Alpharetta, GA) for complete genome sequencing (n=3). The resulting high throughput sequencing reads produced were aligned to a reference *A. mazei* plastome (KY509313) and *rbc*L and *tuf*A sequences and assembled into consensus sequences using the 50% strict criterion in Geneious v. 11.1.5 (Kearse et al., 2012). Phylogenetic analyses were conducted individually for *rbc*L and *tuf*A and the concatenated alignment constructed using the MUSCLE algorithm (Edgar, 2004) in Geneious v. 11.1.5 (Kearse et al., 2012). Model selection (AICc and BIC: GTR+I) and partition scheme (partition 1: 1-562 bp, partition 2: 1-1,123 bp) were determined using PartitionFinder 1.1.0 (Lanfear et al., 2012). Maximum likelihood phylogenetic analysis was conducted using RAxML-HPC2 on XSEDE 8.1.11 (Stamatakis, 2014) for 1,000 bootstrap generations. Bayesian inference was conducted using MrBayes on XSEDE 3.2.2 (Ronquist et al., 2012) for 5×10^6 generations with chain sampling every 1,000 generations with a burnin value of 25% until congruence was met (standard deviation of split frequencies <0.05). Both RAxML and MrBayes were run using the CIPRES Science Gateway (Miller et al., 2010). Species limits were evaluated using the Geneious Species Delimitation Plug-in (Masters et al., 2011) and the resulting P-values (P_{STRICT}, $P_{LIBERAL}$, and Rosenberg's P-value (Rosenberg, 2007)).

5.4 Results

5.4.1 High throughput & Sanger sequencing of type specimens

Summary data for the shotgun sequencing results of the *Avrainvillea amadelpha* (PC42670), *A. lacerata* (BM000515991), and *A. ridleyi* (BM000515995) type specimens are provided in Table 5.3. Complete consensus sequences of the *rbc*L and *tuf*A loci were assembled for the *A. lacerata* and *A. ridleyi* types, but *A. amadelpha* had reduced coverage, resulting in missing data for both loci. Amplification reactions of all extraction controls were unsuccessful, indicating the absence of contamination. Additionally, sequences of all four type specimens are supported as distinct phylogenetic units in the reconstructed phylogeny (Fig. 5.1), further indication that contamination is unlikely.

5.4.2 *Avrainvillea* molecular phylogeny

Sequencing of *tuf*A using the small, overlapping fragment protocol was not successful for all specimens (specifically, the second fragment from ~240bp-395bp), resulting in some specimens having missing data for this portion of the locus. The concatenated *rbc*L+*tuf*A alignment produced a more resolved phylogeny with stronger support values than either individual gene tree and was used for species delimitation analyses. The missing *tuf*A data seemed to confound the resolution of the phylogeny, as the phylogenies produced had very low nodal support values for both maximum likelihood and Bayesian inference reconstructions. Thus, this portion of the gene was removed completely from the alignment, resulting in much higher nodal support values for both analyses (final alignment 1,276 bp). Additionally, while two partitions was recommended by PartitionFinder, analyses without any partition produced higher nodal support values. The phylogeny presented in Fig. 5.1 represents these modifications to the data and analyses. The inclusion of all three genera from the Dichotomosiphonaceae (i.e., *Avrainvillea*, *Cladocephalus* M. Howe, and

Dichotomosiphon A. Ernst) and a diversity of *Avrainvillea* species supported *Avrainvillea*'s previously published polyphyly (Wade et al., 2018); the two clades (A & B) that are supported by the phylogenetic reconstruction are strongly separated with *Cladocephalus* grouping with Clade A (Fig. 5.1). Based on type specimen sequence data, Clade A comprises *A. clavatiramea, A. gardineri,* and a heterotypic type specimen of *A. erecta* (*Chloroplegma papuanum* BM000561613). Non-type specimens collected from type localities support *A. obscura* and *A. riukiuensis* as members of Clade A as well. The species boundaries of *A. nigricans* are still unclear with specimens identified as this taxon grouped with three diverse clades (Fig. 5.2). Furthermore, these clades confound other species boundaries with the inclusion of specimens identified as *A. fulva, A. levis*, and *A. rawsonii*. The identifications of these specimens are considered reliable, as they were provided by D.M. and L.S. Littler and were done so following the completion and publication of their 1992 monograph (B. Brookes, pers. Comm.). The second clade includes *A. amadelpha, A. lacerata*, and *A. ridleyi*, based on type specimen data (Fig. 5.1). Non-type specimen data suggest that *A. calathina*, *A. hollenbergii,* and *A. longicaulis* are also in this clade. This clade corresponds well with the "longicaulis" group described by Olsen-Stojkovich (1985).

5.4.3 Clarification of invasive species identities

Both the Hawai'i and Mediterranean *A. amadelpha* fall into the "longicaulis" group with the *A. amadelpha* type specimen. However, neither clade cluster with the *A. amadelpha* type, which is supported as an early diverging lineage in the group. The Hawai'i species appears to have slight genotypic heterogeneity (max. 1.41% different, up to 18 bp), but is clustering with the *A. lacerata* type specimen (98.75-99.58% similar).

The Mediterranean invasive is clustering with specimens collected from the Red Sea, but not with any of the type specimens that are supported as members of the clade. Species

delimitation strongly supports this clade as reciprocally monophyletic and therefore distinct from the others in the "longicaulis" group.

5.5 Discussion

5.5.1 Morphological plasticity: Caribbean specimens as a case study

The Caribbean *Avrainvillea* specimens are prime examples of the challenges of morphological characters as reliable species delimitation tools for the genus *Avrainvillea*. For example, specimens morphologically identified as *A. levis* grouped into three different sub-clades across clade A (Fig. 5.1) and were identical in sequence to specimens identified as *A. asarifolia*, *A. fulva*, *A. nigricans*, and *A. rawsonii*, which are all similarly polyphyletic. This is particularly interesting because the *A. levis* specimens analyzed in this study were all collected and identified by the same phycologists who described the taxon (Littler and Littler, 1992), suggesting that confident morphological species identification in this genus is elusive to even the most experienced and thorough taxonomic experts. It is also interesting given that *A. rawsonii* was described as being morphologically distinct in lacking a well-developed blade and being composed of knobby, cushion-like projections, further highlighting the degree of phenotypic plasticity observed in *Avrainvillea*. Given its polyphyly, it is possible that this distinctive characteristic is more reflective of environment or ontogeny, as variation in the degree of tightness of blade siphon weaving and blade development has been suggested for other species (Wade et al., 2018).

5.5.2 *Avrainvillea lacerata* in Hawai‘i and the Mediterranean

The phylogeny presented here suggests that the widely reported *Avrainvillea amadelpha* is actually quite rare, and that no specimens included in this study grouped with this early diverging lineage of the "longicaulis" clade (Fig. 5.2). As a result, neither the Hawai‘i nor the Mediterranean species are supported as being *A. amadelpha*. The Hawai‘i "*A.*

amadelpha" grouped closely with the *A. lacerata* isotype specimen and is supported by species delimitation statistics as *A. lacerata* ($P_{IDstrict}$ = 0.67; $P_{IDliberal}$ = 0.90; Rosenberg's P = 1.98x 10^{-3}), thus the record should be revised to reflect this change in identification. The genotypic heterogeneity of the Hawai'i "*A. amadelpha*" suggests multiple arrivals of *A. lacerata* in the MHI: one on the west side of the island, and another on the east side (Fig. 5.2). The grouping of the Hawaiian specimens with specimens from Japan suggests that this location could possibly have provided the source population.

Because none of the type specimens in this study grouped with the Mediterranean invasive *Avrainvillea*, clarification of this species' identification is not possible at this time. The molecular similarity of Red Sea and Mediterranean individuals supports the suggestion by Verlaque et al., (2017) that the alga is most likely a Lessepsian migrant, one of many examples of introduced species to the Mediterranean via the Suez Canal (Bernardi et al., 2010)

5.5.3 A note on the identification of Hawai'i *A.* cf. *erecta*

Inclusion of samples identified as *Avrainvillea obscura* from the type locality (Guam) in our phylogenetic analyses supported their molecular distinction from the Hawai'i *A.* cf. *erecta*, providing clarification on the previously published identification (Wade et al., 2018). *A. erecta* and *A. obscura* share several morphological characteristics, particularly distinctive morphotypes (i.e. mature, well-developed blade vs. immature, loose assemblage of siphons), thus, the identification of the newly introduced species in the MHI was tentative (Wade et al., 2018). While these additional data further supported the identification of this second species in Hawai'i as *A. erecta*, additional work including type specimens of both species are needed to further define the species limits of the *Avrainvillea* "obscura" group.

5.6 Conclusion

This study demonstrates the importance of type specimen sampling and molecular assessment, particularly in algal genera such as *Avrainvillea* that are known to exhibit morphological plasticity. With a modest representation of 7 type specimens of the 37 currently accepted species in the genus, continued work should be done in collaboration with herbaria and museums to build a more robust molecular phylogeny. Additionally, the advent of high throughput sequencing is key to sequencing highly degraded DNA and should be targeted for further type specimen work.

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5.10 Tables

Table 5.1. Specimen collection and accession information.

Species	Field/Herbarium Code	Collection Location	Collection Date	Collector	Determiner	*rbc*L	*tuf*A
Avrainvillea lacerata	ARS00297	Ko‘olina, O‘ahu, Hawai‘i; 40ft	3-May-2005	H. Spalding	R. Wade	X	X
Avrainvillea cf. *rawsonii*	ARS08486	Graham's Harbor, San Salvador, the Bahamas;	24-May-13	A. Carlisle	A. Carlisle	X	X

		in sediment near Thalassia					
Avrainvillea cf. *amadelpha* A. Gepp & E.S. Gepp	ARS08611; GH0011213, GUAM 00625	Okinawa, Konbu Beach to Tengan [sic], Japan; LAT 26.405934, LON 127.84378	29-Jun-2010	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *nigricans*	BDA0333					X	X

Avrainvillea cf. *amadelpha* A. Gepp & E.S. Gepp	BISH540911	Luzon, Philippines	8-Mar-1968	G.T. Kraft	J. Olsen-Stojkovich	X	
Avrainvillea cf. *amadelpha* A. Gepp & E.S. Gepp	BISH540912	Palmyra Island, Palmyra Atoll	28-Dec-1959	H. Möller	J. Olsen-Stojkovich	X	
Avrainvillea cf. *nigricans*	BISH589944	Nameless Island, Hamilton Sound	7-Jul-1925	A. Hof	A. Hof	X	X
Avrainvillea clavatiramea TYPE	BM000515983	Victoria, Australia	10-Dec-1887	J.B. Wilson	E. Gepp & A. Gepp		X
Avrainvillea gardineri TYPE	BM000515984	Mauritius, Agalega Islands,	1905	J.S. Gardiner		X	X

		LAT -20.278000, LON 57.597000					
Avrainvillea lacerata TYPE	BM000515991	Friendly Islands		W.H. Harvey	J. Agardh	X	X
Avrainvillea ridleyi TYPE	BM000515995	Christmas Island LAT-10.480056, LON 105.628008	1-Oct-1904	H.N. Ridley	E. Gepp & A. Gepp	X	X
Avrainvillea riukiuensis	BM000569543	Rykyy Shoto,	14-Dec-1994	H. Ohba	Yamada	X	X

		Japan, LAT 26.7386 LON 128.2058					
Avrainvillea cf. asarifolia	DML30402	W. Culebra, Puerto Rico	4-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	
Avrainvillea cf. asarifolia	DML30517	Prickly Pear Cays, Anguilla	6-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *asarifolia*	DML30612	Base Grande Case - N,	7-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X

		St. Maarten					
Avrainvillea cf. *asarifolia*	DML30670	Anse de Colmbier, St. Barthelemy	8-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. geppiorum	DML30810	Rocher la Perle, Martinique	13-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *longicaulis*	DML30949	Rocher la Perle, Martinique	13-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X

Avrainvillea cf. *nigricans*	DML30961	Diamond Rock, Martinique	14-Jun-95	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *rawsonii*	DML31248	Petite St. Vincent & Martinique, St. Vincent	19-Jun-1995	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *levis*	DML67780	Twin Cays Grouper Gardens, Belize	25-Mar-2006	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X

Avrainvillea cf. *levis*	DML67882	Twin Cays Hidden Lake, Belize	26-Mar-2006	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *levis*	DML67883	Twin Cays Grouper Gardens, Belize	25-Mar-2006	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X
Avrainvillea cf. *levis*	DML67884	Twin Cays Grouper Gardens, Belize	25-Mar-2006	D.S. & M.M. Littler	D.S. & M.M. Littler	X	X

Avrainvillea cf. *amadelpha* A. Gepp & E.S. Gepp	F4_LLG97	Kerkennah Archipelago, Tunisia		M. Verlaque	M. Verlaque		X
Avrainvillea cf. *obscura*	GLS031A	Agat Bay, Guam	27-Feb-1977	G.L. Stillberger	G.L. Stillberger	X	X
Avrainvillea cf. *erecta*	GWS025746	Roxas Port, Roxas, Oriental Mindoro, Philippines, LAT 12.58464	8-Dec-2010				X

		5, LON 121.523236, 2M deep on cobble					
Avrainvillea cf. *nigricans*	GWS029535	Victoria, Australia, LAT -38.34245 LONG 141.6075; Subtidal in sand, ~3M	15-Nov-2011				X
Avrainvillea sp.	HEC15984	Kalpitiya, Sri Lanka					X

Avrainvillea cf. *amadelpha* A. Gepp & E.S. Gepp	HEC16015	Dickwell a, Sri Lanka					X
Avrainvillea cf. *rawsonii*	HV00892	Jamaica		H. Verbruggen			X
Avrainvillea cf. *clavatiramea*	HV04012	Victoria, Australia		H. Verbruggen			X
Avrainvillea sp. 1	HV04061	Apo Island, Philippin es		H. Verbruggen			X
Avrainvillea amadelpha	PC0042670	Mauritius , Agalega Islands		Leduc	J. Olsen-Stojkovich	X	X
Avrainvillea sp.	PNCI16_MP29	Pakin, Pohnpei, 50 m	1-Aug-2016	S. Rowley		X	X

Avrainvillea cf. *obscura*	RT2019A	MLT, Pago Bay, Guam	16-Apr-1968	R.Tsuda	R. Tsuda	X	X
Avrainvillea cf. *fulva*	SE6463	Summerland Key, FL, USA	18-Aug-1964	S. Earle, C.J. Dawes		X	X
Avrainvillea cf. *amadelpha*	TS1337	Sonai: Iriomote Site1, 1-2m	24-Jul-2012	T. Sauvage	T. Sauvage		X
Avrainvillea sp.	TS1683	Smithsonian Aquarium Museum		T. Sauvage			X

Avrainvillea cf. asarifolia	TS2044					X	X

Table 5.2. Primer information and reference.

Primer Name	Primer Sequence (5' → 3')	Primer Length (bp)	T_M (°C)	Reference
rbcLF	AAAGCNGGKGTWAAAGAYTA	20	50.3	Pierce et al., (2006)
rbcL223R	KTCTTCACCDGCDACTGGTT	20	56.2	Wade et al., (2018)
rbcL204F	GAACCAGTHGCHGGTGAAGA	20	56.3	Wade et al., (2018)
rbcL396R	GWGGHCCTTGRAAHGTTTT	19	51.1	Wade et al., (2018)
rbcL381F	ACRTTTCAAGGVCCACCACA	20	57.7	Wade et al., (2018)
rbcLR	CCAWCGCATARANGGTTGHGA	21	56.1	Pierce et al., (2006)
tufA_alg_up	ATGATWACNGGHGCNGCWCAATGG	25		Händeler et al., (2010)
tufA236R	AGCRTTTAAAGCWGATCCDGC	21	55.5	This study
tufA216F	GCHGGATCWGCTTTAAARGCT	21	55.3	This study
tufA411R	CYGTRCCACGACCWGTAATM	20	54.7	This study
tufA392F	KATTACWGGTCGTGGYACRG	20	54.7	This study
tufA607R	ACCATTCCRCKCTGAAYATC	20	53.8	This study

tufA588F	GATRTTCAGMGYGGAATGGT	20	53.8	This study
tufA_alg_do	GACCWCAATTTTATGTTMGAACAA	24		Händeler et al., (2010)

Table 5.3. High throughput sequence data information for type specimens. *minimum coverage at sites with coverage; some sites did not have any coverage, resulting in missing data.

Accession	Species	Number of Raw Reads	#of reads mapped to reference plastome	# of reads mapped to reference *rbc*L	Minimum coverage	Maximum coverage	# of reads mapped to reference *tuf*A	Minimum coverage	Maximum coverage
BM000515995	*Avrainvillea ridleyi*	17,020,240	744,707	6,477	320	890	9,084	279	1,089
BM000515991	*Avrainvillea lacerata*	22,696,658	80,568	823	38	165	1,266	48	207
PC42670	*Avrainvillea amadelpha*	31,275,664	41,461	528	2*	111	432	11*	85

5.11 Figures

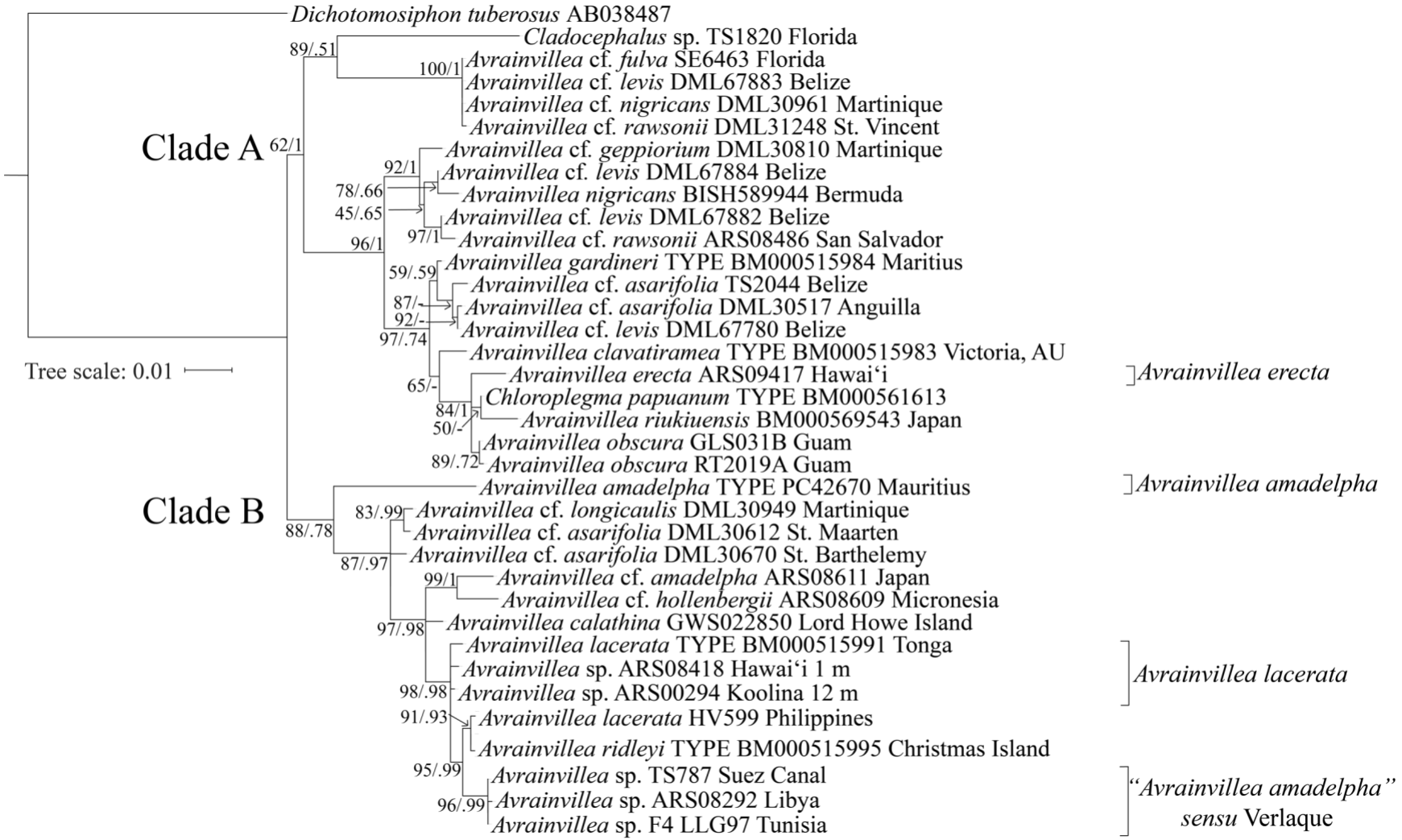
Dichotomosiphon tuberosus AB038487
Cladocephalus sp. TS1820 Florida
89/.51
100/1
Avrainvillea cf. *fulva* SE6463 Florida
Avrainvillea cf. *levis* DML67883 Belize
Avrainvillea cf. *nigricans* DML30961 Martinique
Avrainvillea cf. *rawsonii* DML31248 St. Vincent
Clade A
62/1
Avrainvillea cf. *geppiorium* DML30810 Martinique
92/1
Avrainvillea cf. *levis* DML67884 Belize
78/.66
Avrainvillea nigricans BISH589944 Bermuda
45/.65
Avrainvillea cf. *levis* DML67882 Belize
97/1
96/1
Avrainvillea cf. *rawsonii* ARS08486 San Salvador
Avrainvillea gardineri TYPE BM000515984 Maritius
59/.59
Avrainvillea cf. *asarifolia* TS2044 Belize
87/-
Avrainvillea cf. *asarifolia* DML30517 Anguilla
92/-
Avrainvillea cf. *levis* DML67780 Belize
97/.74
Avrainvillea clavatiramea TYPE BM000515983 Victoria, AU
Tree scale: 0.01
65/-
Avrainvillea erecta ARS09417 Hawai‘i
Avrainvillea erecta
Chloroplegma papuanum TYPE BM000561613
84/1
Avrainvillea riukiuensis BM000569543 Japan
50/-
Avrainvillea obscura GLS031B Guam
89/.72
Avrainvillea obscura RT2019A Guam
Avrainvillea amadelpha TYPE PC42670 Mauritius
Avrainvillea amadelpha
Clade B
83/.99
Avrainvillea cf. *longicaulis* DML30949 Martinique
88/.78
Avrainvillea cf. *asarifolia* DML30612 St. Maarten
87/.97
Avrainvillea cf. *asarifolia* DML30670 St. Barthelemy
99/1
Avrainvillea cf. *amadelpha* ARS08611 Japan
Avrainvillea cf. *hollenbergii* ARS08609 Micronesia
97/.98
Avrainvillea calathina GWS022850 Lord Howe Island
Avrainvillea lacerata TYPE BM000515991 Tonga
Avrainvillea sp. ARS08418 Hawai‘i 1 m
Avrainvillea lacerata
98/.98
Avrainvillea sp. ARS00294 Koolina 12 m
91/.93
Avrainvillea lacerata HV599 Philippines
Avrainvillea ridleyi TYPE BM000515995 Christmas Island
95/.99
Avrainvillea sp. TS787 Suez Canal
96/.99
Avrainvillea sp. ARS08292 Libya
Avrainvillea sp. F4 LLG97 Tunisia
“*Avrainvillea amadelpha*”
sensu Verlaque

Figure 5.1. Maximum likelihood reconstructed *Avrainvillea* phylogeny of the concatenated rbcL and tufA dataset (1276 bp). Support values represent bootstrap values/posterior probabilities. Scale bar = number of substitutions per site.

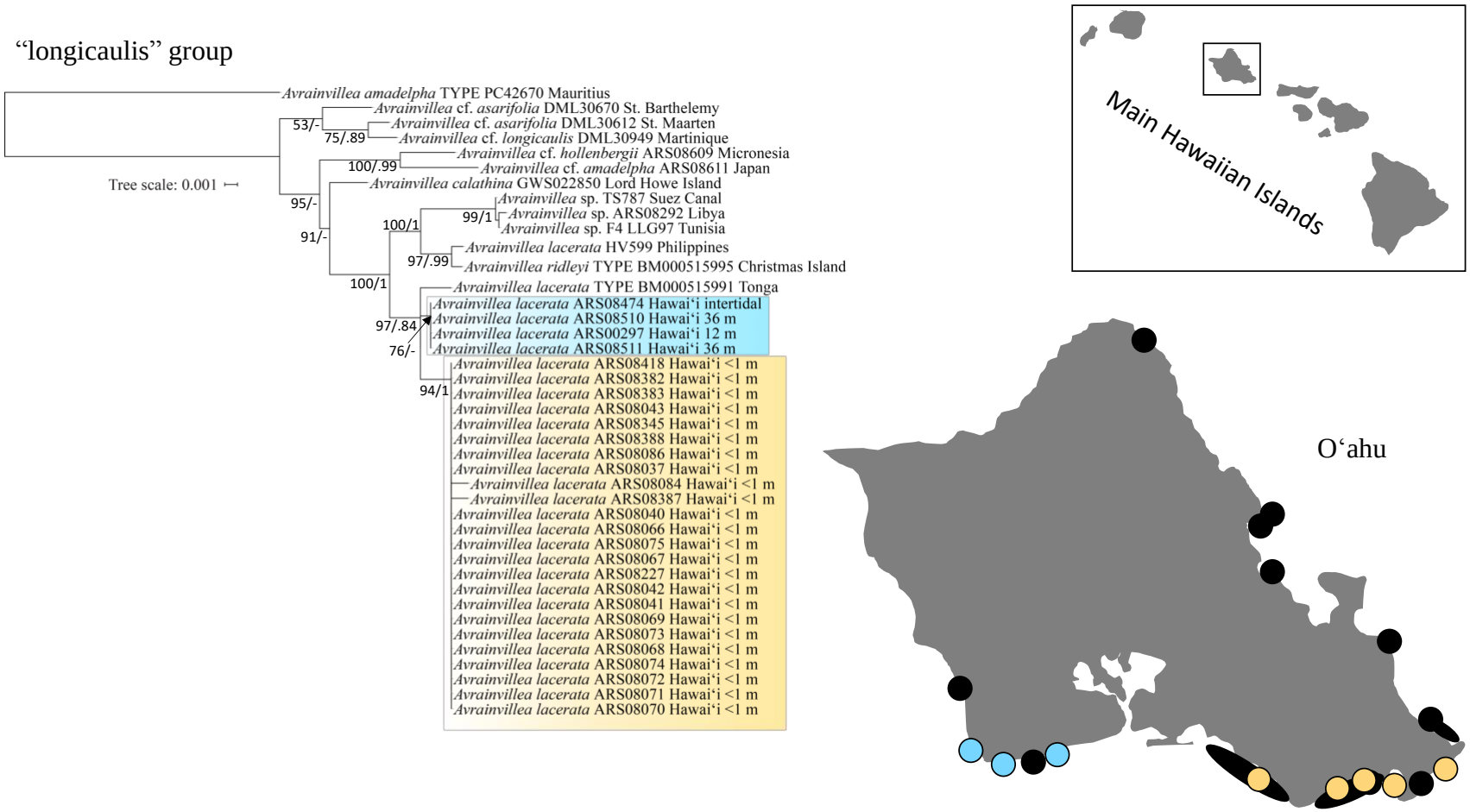

Figure 5.2. Maximum likelihood reconstructed *Avrainvillea* phylogeny of the concatenated rbcL and tufA dataset (1276 bp) for the "longicaulis" group in relation to *Avrainvillea lacerata* populations around the island of O'ahu in the Main Hawaiian Islands. Support values represent bootstrap values/posterior probabilities. Scale bar = number of substitutions per site.

CHAPTER 6. A new record of *Avrainvillea* cf. *erecta* (Berkeley) A. Gepp & E. S. Gepp (Bryopsidales, Chlorophyta) from urbanized estuaries in the Hawaiian Islands

Rachael M. Wade, Heather L. Spalding, Kimberly A. Peyton, Kevin Foster, Thomas S. Sauvage, Matthew Ross, Alison R. Sherwood

6.1 Abstract

A second species in the siphonous green algal genus *Avrainvillea* was recently discovered off the island of Oʻahu in the Main Hawaiian Islands. Specimens were collected from Honolulu Harbor, including its entrance channel, and near Keʻehi Harbor. These locations are both in Mālama Bay on Oʻahu's south shore in or adjacent to urbanized estuaries, respectively. *In situ* observations, morphological and molecular assessments were conducted to examine the alga's habit and distribution, as well as to assess its putative species identification. The alga occurred in sand as single individuals or in clusters of several individuals at both sites, and near or within seagrass beds (*Halophila decipiens*) and algal meadows composed of the green alga *Halimeda kanaloana* and an unidentified *Udotea* species at the Keʻehi Harbor site. All analyses supported both populations as representative of the same taxa, reported until further investigation in the broad Pacific as *Avrainvillea* cf. *erecta* based on morphological and molecular analyses. This record of a second *Avrainvillea* species in Hawai'i is of particular concern considering that an alga recognized as *A. amadelpha*, first observed in 1981 from two locales on Oʻahu's south shore, has become invasive in Hawaiʻi's intertidal to mesophotic environments.

Keywords: *Avrainvillea*, Bryopsidales, Chlorophyta, estuary, Hawaiʻi, *rbc*L, seagrass, *tuf*A

6.2 Introduction

The siphonous green algal order Bryopsidales includes over 500 extant species (Guiry and Guiry 2017). This diversity is largely due to the evolution of unique traits, which allows them to become significant and persistent members of the marine environment. These characters include macroscopic unicellularity (Vroom and Smith 2001), rapid growth

(Smith et al. 2004), adaptability to low nutrient environments (Lobban and Harrison 1994, Smith et al. 2004, Malta et al. 2005), chemical defenses (unpalatability and subsequent escape from predators, e.g. Hay et al. 1987, Becerro et al. 2001, Baumgartner et al. 2009), and vegetative reproductive ability via fragmentation (Hillis-Colinvaux et al. 1965, Walters and Smith 1994, Vroom et al. 2003, Wright and Davis 2006). The ecological success of some members of the Bryopsidales when introduced to new environments has been strongly demonstrated by the invasion and persistence of *Caulerpa taxifolia* (M. Vahl) C. Agardh in the Mediterranean (Meinesz et al. 2001) and *Codium fragile* ssp. *tommentosoides* (van Goor) P. C. Silva (= ssp. *fragile* (Suringar) Hariot) across the globe (Provan et al. 2004).

An unknown species of *Avrainvillea* was first documented on the western shore of O‘ahu in 1981. By 1985, the alga had spread to the inter- and subtidal environments of O‘ahu’s south shores. This distribution was documented by Brostoff (1989), who identified the alga as *Avrainvillea amadelpha*(Montagne) A. Gepp & E. S. Gepp. However, Brostoff 1989 stated that the alga also closely resembled three other species; more recent morphological and molecular analyses have also not been able to conclusively identify the alga to the species level because of its morphological plasticity, but *A. amadelpha* is most likely incorrect (Wade et al. 2015). Thus, throughout the manuscript this species will be referred to provisionally as “*A. amadelpha*”. Interestingly, an invasive alga identified as "*A. amadelpha*" was recently recorded in the Mediterranean as well (Verlaque et al. 2017). More recently, a population of a second *Avrainvillea* species, distinct in habit from "*A. amadelpha*", was discovered on October 14-16, 2014 in Honolulu Harbor, including its entrance channel and turning basin, from 12-15 m depths (Fig. 1). Honolulu Harbor is the

principal seaport for all of the Hawaiian Islands, handling approximately 80% of goods imported into the islands and servicing both international and domestic vessels. The alga was found again on April 22, 2017 seaward of Keʻehi Lagoon from 25-40 m depths; this area is near the Keʻehi Boat Harbor in the vicinity of offshore anchorages for large commercial vessels, as well as an urbanized and commercialized area of Honolulu. Both sites are located in Mālama Bay on the south shore of Oʻahu in the Main Hawaiian Islands (Fig. 1). Here we provide *in situ* observations of these populations in urbanized estuaries, and assess the molecular identity of the new species record and its morphology in comparison to the previously reported "*A. amadelpha*."

6.3 Methods

6.3.1 *In situ* observations

A quantitative seagrass community survey using SCUBA from 12-18m depth was conducted jointly by the U.S. Fish and Wildlife Service and State of Hawaiʻi Department of Land and Natural Resources - Division of Aquatic Resources in Honolulu Harbor from October 14-16, 2014 as part of regular benthic surveys in preparation of scheduled dredging. Field data were collected in the planned dredge footprint at eight locations within the turning basin and entrance channel, locations which are referred to as "Impact Sites" (Appendix r). An additional eight sites were sampled outside of the dredging areas, referred to as "Control Sites." Five-minute swims were made at each site to record other benthic species, during which time a population of *Avrainvillea* was discovered. After this unexpected discovery, rapid assessments and collections of this alga were undertaken at each site where it was observed.

Specimens were also discovered on April 22, 2017 offshore of Ke‘ehi Lagoon, south O‘ahu using SCUBA at 25-40 m depths. Subsequent qualitative SCUBA surveys were conducted at 20-30 m depths at three sites near the original collection site on May 18, 2017 to make qualitative observations regarding its habitat and associated organisms.

Specimens were collected on 22 April 2017 offshore of Ke‘ehi Lagoon, south O‘ahu (21°17.4’N; 157°55.23’W) using SCUBA at 25-40 m depths. Subsequent qualitative SCUBA surveys were conducted at 20-30 m depths at three sites near the original collection site on 18 May 2017 to make qualitative observations regarding its habitat and associated organisms.

6.3.2 Morphological characterization

Two specimens collected in 2014 (BISH768338-9) and six collected in 2017 (BISH768278-83) that included what appeared to be mature and juvenile forms or possibly ecotypes were selected for morphological and molecular characterization (Appendix 5). Additional specimens provided by co-authors and collaborators were also assessed for species identification in the same manner (Appendix 5). Morphology was evaluated using 12 macroscopic characters and 21 microscopic characters (Appendix 6). Tentative species-level identification was determined by comparison with original species descriptions (Decaisne 1842, Agardh 1887, Gepp and Gepp 1908, Gepp and Gepp 1911, Olsen-Stojkovich 1985, Littler and Littler 1992) and the re-evaluated descriptions and dichotomous keys provided by Olsen-Stojkovich (1985) and Littler and Littler (1992). In particular, the groups described by Olsen-Stojkovich (1985) that are a result of similarity-graph clustering using morphological characters and growth habit (i.e. the “longicaulis”, “nigricans”, and “*obscura*” groups) were used to compare the newly recorded *Avrainvillea* sp. and the previously recorded “*A. amadelpha*”.

6.3.3 Molecular assessment

In addition to the specimens used for morphological assessment, two type specimens of heterotypic synonyms of *Avrainvillea erecta* (*Chloroplegma papuanum* Zanardini and *Rhipilia andersonnii* G. Murray) were borrowed from the Natural History of Museum of London and included in our molecular assessment (morphological assessment, and therefore additional destructive sampling, was not permitted). DNA extraction was completed using the OMEGA E.Z.N.A® Plant DNA Kit (OMEGA bio-tek, Norcross, GA U.SA.). For the two type specimens, the protocol developed by Hughey et al. (2001) was used. DNA extracts were amplified for portions of two chloroplast gene regions: the 5' end of *rbc*L (ribulose-1,5-bisphosphate carboxylase/oxygenase – large subunit, 562 bp) and *tuf*A (elongation factor Tu, 714 bp). These gene regions were selected as informative and reliably sequenced regions for siphonous green algae (Leliaert et al. 2014) and their use in previous Bryopsidales phylogenetic studies (e.g. Curtis et al. 2008, Verbruggen et al. 2009a, Verbruggen et al. 2009b, Wade and Sherwood 2017, Wade and Sherwood 2018). For the type specimens, a modified protocol was used with short, overlapping fragments for each gene, rather than amplifying the entire fragment at once; a new protocol was developed for *rbc*L and the *tuf*A protocol described by Sauvage et al. 2014 was used. For *rbc*L, three fragments were amplified using the forward (rbcLF) and reverse (rbcLR) primers developed by Pierce et al. (2006) and newly developed internal primers rbcL223R (5' KTCTTCACCDGCDACTGGTT 3'), 204F (5' GAACCAGTHGCHGGTGAAGA 3'), 400R (5' GWGGHCCTTGRAAHGTTTT 3'), and 381F (5' ACRTTTCAAGGVCCACCACA 3'). Sequences were edited and aligned with previously generated sequences and reference sequences available on GenBank using Geneious 7.1.8 (BioMatters, Auckland, N.Z.). The two gene alignments were then concatenated for phylogenetic analyses. Model selection

(AICc and BIC: GTR+I) and partition scheme (no partitions) were determined using PartitionFinder 1.1.0 (Lanfear et al. 2012). Maximum likelihood phylogenetic reconstruction was conducted using RAxML-HPC2 on XSEDE 8.1.11 (Stamatakis 2014) for 1,000 bootstrap generations. Bayesian inference was conducted using MrBayes on XSEDE 3.2.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) for 2 x 10^6 generations with chain sampling every 1,000 generations and a burnin value of 25% until congruence was met (standard deviation of split frequencies <0.05). Both RAxML and MrBayes were accessed on the CIPRES Science Gateway (Miller et al. 2010).

6.4 Results

6.4.1 *In situ* observations

During the 2014 seagrass community survey, the newly discovered *Avrainvillea* sp. was observed at six of 16 survey sites in the Honolulu Harbor entrance channel from 12-15m depths (four "Control Sites", two "Impact Sites"; Appendix 4). The two morphologies (blade-like versus assemblage of loose siphons) differed in their exposure to water flow – individuals with a completely formed blade were often elevated and fully exposed to water motion, while individuals with a loose assemblage of siphons were in depressions or divots and therefore protected (K. Peyton, unpublished data). This water motion effect was also supported by informal experimentation: in water tables without water flow, blades were observed to unweave and become loose assemblages while specimens with water flow maintained the blade morphology (K. Peyton, unpublished data).

In 2017, the newly recorded *Avrainvillea* sp. was observed as single individuals or in patches with 10-20 individuals per m^2 (estimated visually) in areas with deep sand (Fig. 2a, b). The dominant vegetation in these sand beds was the seagrass *Halophila decipiens* Ostenfeld,

patches of the macroalga *Halimeda kanaloana* Vroom, and an unidentified *Udotea* sp. Several individuals were observed with feeding scars (large bite marks), giving some thalli a U-shaped appearance. The holdfasts of larger, more mature individuals protruded from the sediment by approximately 1-5 cm, creating a conical mound at the base of the alga. Individuals were generally clean and not heavily epiphytized. The two morphologies at this location experienced very little water motion due to attenuation of wave motion with depth, and therefore were most likely the result of differences in age. The individuals with spherical assemblages of loose siphons were consistently much smaller in thallus size than the well-formed blade morphology.

6.4.2 Morphological characterization

The specimens were olive-green upon collection and dried to a darker green with fulvous, or tawny, coloration (Fig. 2c, d). Specimens were categorized as mature adults (BISH768278), immature adults (BISH768279-80) or juveniles (BISH768281-3). Adult individuals, both mature and immature, ranged in overall length from 6.7-15.8 cm; frond length ranged from 3.6-10.8 cm. Each adult thallus was distinctly differentiated into a holdfast, stipe, and blade. The rhizomatous holdfasts comprised up to 46% of the total thallus length. The thin stipes of adult individuals supported a lightly zonate and reniform to sub-reniform blade; margins appeared to be composed of loose aggregates of siphons, but not necessarily lacerate. Individuals that appeared to be juveniles consisted only of a holdfast and a spherical assemblage of loose siphons, which appeared to be in the beginning stages of forming a blade. Siphons throughout the specimens (e.g. margin, blade, stipe, and holdfast) were mostly cylindrical to slightly torulose and measured in width 11.1-(25.4-59.1)-93.1 µm with acute and deep constrictions above the dichotomies (Fig. 2e). Constrictions were also common below the dichotomy, except in the holdfast siphons. Apices were primarily rounded,

but also rarely blunt or sub-clavate. Siphons appeared olive green, transparent, or fulvus, which was attributed to overall siphon color and/or chloroplast pigmentation. These characters and measurements suggest affinity with the description of *Avrainvillea erecta* (Berkeley) A. Gepp & E.S. Gepp and their further morphological characterization by Olsen-Stojkovich (1985).

6.4.3 Molecular assessment

The majority of examined specimens were sequenced for both *rbc*L and *tuf*A, however, molecular characterization of historical material was only successful for *rbc*L for one of the heterotypic synonym type specimens - *Chloroplegma papuanum* BM000561613. The concatenated alignment of the two gene regions yielded a dataset of 1,360 bp. Both the Maximum Likelihood and Bayesian inference phylogenetic reconstructions strongly supported that the newly recorded *Avrainvillea* species, *A.* cf. *erecta,* was clearly distinct from Hawai‘i specimens identified as “*A. amadelpha*” (Brostoff 1989); these newly sequenced specimens belong to the “*obscura*” group while “*A. amadelpha*” clusters within the “longicaulis” group (Olsen-Stojkovich 1985) (Fig. 3). These analyses also support the monophyletic grouping of sequences from the newly sequenced specimens and those morphologically identified as *A.* cf.*erecta* (Berkeley) A. Gepp & E. S. Gepp from Japan and Micronesia. Although they exhibited two different morphs (loose siphons or blade), all specimens from the two Hawai‘i populations had identical DNA sequences.

6.5 Discussion

The morphological and molecular characterization of the newly recorded *Avrainvillea* species showed most affinities to the description of *A. erecta* based on stipe length, blade habit, siphon width and morphology (constriction at dichotomy); however

considering that we could not obtain material from type locality or the basionym type specimen (*Dichonema erectum* Berkeley 1842), we temporarily consider the newly recorded species as *A.* cf. *erecta* until further research can be conducted (Suppl. material 2, Fig. 3). For instance, the specimens closely resemble *A. obscura* (C.Agardh) J.Agardh, in part due to the description of the species' ecomorphs that resemble both morphologies described here (Agardh 1887). However, a reduced stipe undifferentiated from a cuneate blade, general lack of blade zonation, and non-fulvous siphons of *A. obscura* make it a less likely match than *A. erecta*. The phylogenetic separation of the Hawai'i specimens and the *Chloroplegma papuanum* specimen does confuse the issue because *C. papuanum* type is a heterotypic synonym of *A. erecta*. However, the undifferentiated stipe and blade of the specimen, and cuneate, non-zonate blade (Fig. 2f), which closely matches the gross morphological description of *A. obscura*, suggests that this specimen is not truly representative of *A. erecta* and is possibly wrongly synoymized with it. Therefore, we maintain our conclusion that the specimens recovered in Hawai'i should be regarded as *A.* cf. *erecta* for the time being.

Avrainvillea cf. *erecta* was not observed at the Honolulu Harbor sites when it was resurveyed in March 2016, and reduced *Halophila decipiens* cover was found as compared to 2014. This is most likely due to scheduled dredging that occurs approximately every 15 years. Interestingly, *Halimedakanaloana* was observed at one site where it was absent two years previously. Soft bottom assemblages in the urbanized estuary are subject to disturbance, including naturally occurring factors like storms as well as anthropogenic forces like dredging that result in light attenuation (Ruffin 1998). *Halophila decipiens* recovery from such disturbances is dependent on its seed bank when there is complete loss of its vegetative canopy

(McMillan 1988). Similarly, due to its robust holdfast, it is possible that *A.* cf. *erecta* could persist below the surface of the benthos and could regrow from holdfast siphons (Hillis-Colinvaux et al. 1965, DeWreede 2006), therefore this high traffic area and benthos should continue to be monitored regularly.

The site examined near Keʻehi Lagoon has historically been dominated by *H. decipiens* (M. Ross, unpublished data). However, during the past two years, *H. kanaloana* has begun to appear, and in many places, is now one of the dominant species. Similarly, *Udotea* sp. was only observed for the first time in the area earlier in 2017. Based on these observations, this habitat may be undergoing significant shifts in species composition, in which *A.* cf. *erecta* is playing a part (M. Ross, unpublished data).

The morphological record for *A. erecta* (which may include genetically divergent cryptic diversity and is thus to be considered carefully) encompasses the East coast of Africa and the Red Sea to as far as the western Pacific in the waters of New Zealand and several Pacific Islands (Guiry and Guiry 2017). Given the proximity of the newly recorded Oʻahu populations to major ports and harbors, and intensity of boat traffic reaching these harbors, it is likely that the alga (e.g. as fragments) was transported here via solid ballast (Carlton 1987), sea chest, or anchor entanglement; hull fouling is an unlikely vector (unless heavily fouled to provide sufficient microhabitat) due to the normal environment and growth habit of the alga as a psammophytic species with rhizomatous holdfast.

Alternatively, the alga could have arrived as a result of Pacific currents; the Pacific Gyre carries water from Southeast Asia and Japan through the Pacific Ocean north of the Hawaiian Islands to California, and returns to the East Pacific south of the Hawaiian Islands. Additionally, the Equatorial Countercurrent feeds into the gyre, supplying it with water from

Australia, New Zealand, and the Pacific Islands (Tomczak and Godfrey 1994). Introduction to Hawai‘i as a result of the 2011 Tohoku earthquake and subsequent tsunami in Japan are possible; indeed, studies have recently recorded introduced algal species on tsunami debris on the west coast of North America and in Hawai‘i (e.g. West et al. 2016, Carlton et al. 2017, Carlton et al. 2018, Hanyuda et al. 2018). Given the vegetative propagation achieved by members of the Bryopsidales, it is possible that very small fragments were carried to Hawai‘i by one means or another naturally (Hillis-Colinvaux et al. 1965, Walters and Smith 1994, Vroom et al. 2003, Wright and Davis 2006). Additionally, the close identity of DNA sequences (3-8 bp differences) obtained for the newly recorded specimens with those from Japan and Micronesia would suggest their geographical origin from the western Pacific; however, population genetic work is needed to conclusively demonstrate this connection.

Given that *A. erecta* was originally described from specimens collected from 15-36 m (Gepp and Gepp 1911), and additional records indicate that this species is also common in the intertidal to shallow subtidal (Natural History Museum 2017), this new record for the Hawaiian Islands is of serious concern, especially considering the prevalence and impacts of "*A. amadelpha*" in Hawai‘i and the "*A. amadelpha*" recently recorded in the Mediterranean Sea (Verlaque et al. 2017). "*A.amadelpha*" now inhabits the intertidal, subtidal, and mesophotic environments in Hawai‘i (Spalding 2012), and is considered invasive (Peyton 2009, Cox et al. 2017), altering the benthic ecosystem (Martinez et al. 2009), and competing with native species (Peyton 2009). Given these characteristics, considerable attention should be given to *A. erecta* in the Hawaiian Islands to monitor its possible expansion and competition with other psammophytic phototrophs in order to allow timely mitigation strategies if needed. Furthermore, these areas of high anthropogenic disturbance combined

with the ecological success of this siphonous green algae makes this genus a concern for continued introductions worldwide, especially harbors experiencing heavy maritime traffic like Honolulu.

6.6 Acknowledgements

We thank Drs. Gerald Kraft, Daryl Lam, Chris Lane, Gary Saunders, and Tom Schils for provision of both specimens and taxonomic expertise, as well as the Natural History Museum of London for their generous loan of type specimens. We also thank Yue Tang for her early work on "*A. amadelpha*" and Paul Murakawa for diving and field collection support. Additional thanks to Drs. Anthony Amend, Patrick Krug, Daniel Rubinoff, and Celia Smith for their continued support and advice.

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6.8 Figures

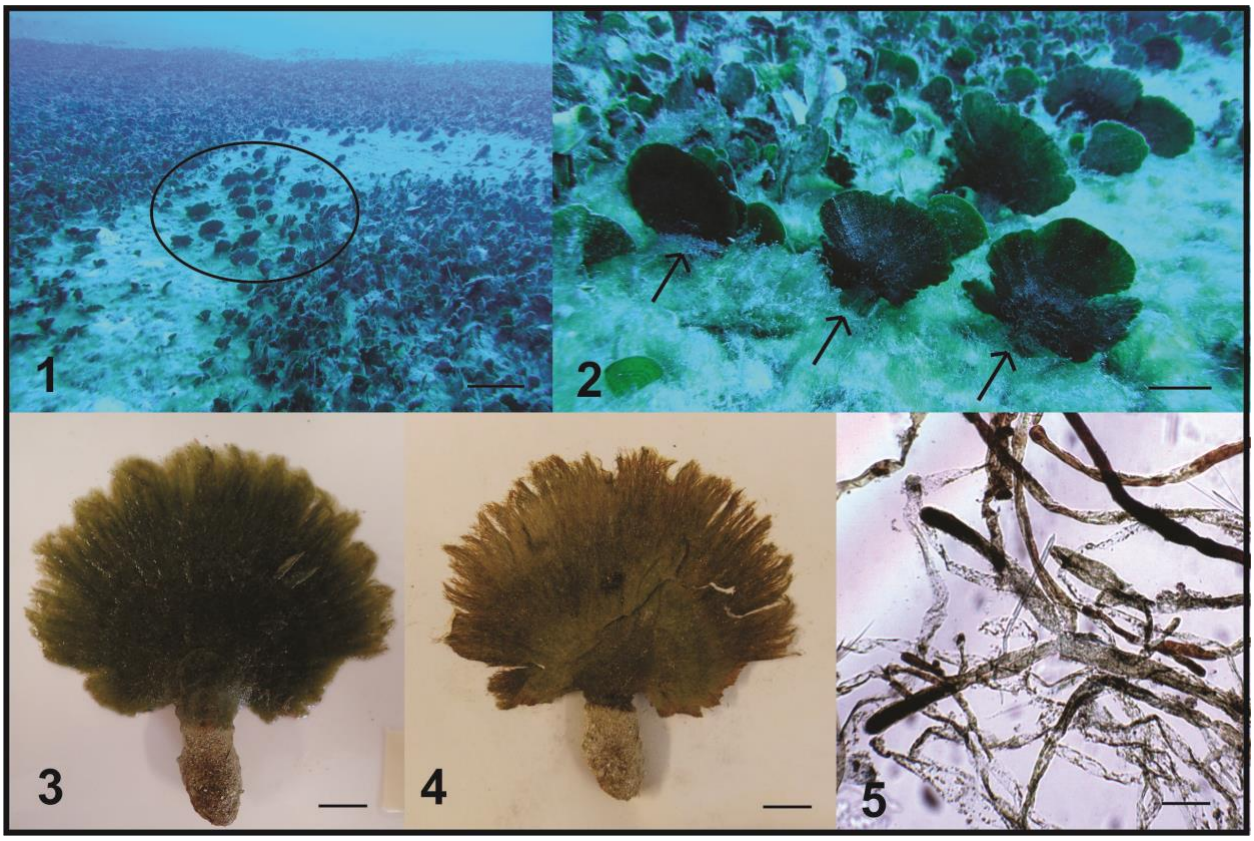

Figure 6.1. *Avrainvillea erecta* habit and diagnostic features from O‘ahu in the Main Hawaiian Islands. **A** *Avrainvillea erecta* (indicated by circle) within a dense bend of other siphonous autotrophs. **B** *Avrainvillea erecta* (indicated by arrow) amongst other psammophytic macroalgae, including the green alga *Udotea* sp. and *Halimeda kanaloana*. **C** Fresh specimen of *Avrainvillea erecta* (BISH768278) exhibiting the slightly eroded margin of loose siphons, sub-reniform blade, and well-developed holdfast. Scale bar = 1cm. **D** Dried specimen of *Avrainvillea erecta* (BISH768278) exhibiting the fulvous coloration and light radial zonation. Scale bar = 1cm. **E** Siphons showing cylindrical to slightly torulose shape, acute constriction above the dichotomy, and fulvous coloration, particularly towards the apices. Scale bar = 50µm.

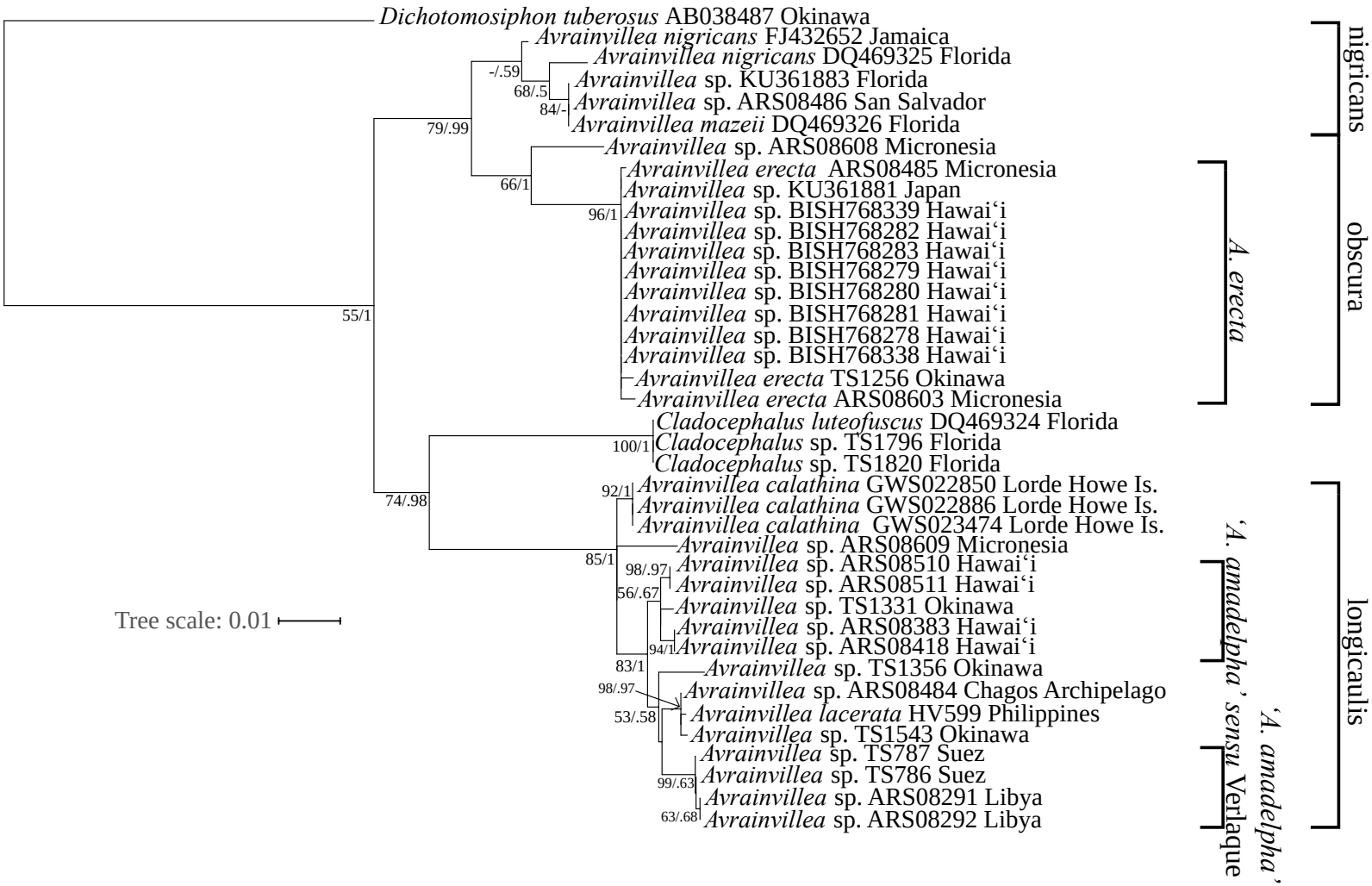

Figure 6.2. Maximum Likelihood-estimated *Avrainvillea* phylogeny from the rbcL and tufA concatenated alignment. The first clade notations specify the two *Avrainvillea* spp. found in Hawai‘i, second clade notations specify the *Avrainvillea* groups

described by Olsen-Stojkovich (1985). Scale bar = substitutions per site. Nodal support values represent maximum likelihood bootstrap values and Bayes inference posterior probabilities, respectively.

CHAPTER 7. CONCLUSIONS

7.1 Limitations and Future Directions

7.1.1 Kleptoplastidic sea slugs

This dissertation provides a thorough examination of the diversity of and limitations of host selection for *Plakobranchus* cf. *ianthobapsus*. However, it is important to keep in mind that *Plakobranchus* is a species complex comprising at least 10 species (Krug et al., 2013). Given the variability in kleptoplast source diversity among other Sacoglossa genera such as *Elysia* (Becerro et al., 2001; Curtis et al., 2006; Jensen, 1997; Middlebrooks et al., 2014), it is unlikely that the kleptoplast source diversity reported here is completely representative of that for other species or even other populations of *Plakobranchus*. Furthermore, it is unlikely that the algal community, and therefore the diversity of algal host species, reported here are the same for other regions and populations. Thus, exploration of these other species may continue to reveal additional cryptic alga diversity and data with diverse applications e.g. algal community assemblages, herbivore ecology, and invasive species detection.

The question that still remains largely unexplored is whether kleptoplast diversity reflects the diversity of algae utilized by sacoglossans. The absence of gut contents due to sacoglossan feeding style (i.e. “sap sucking” of cytoplasm) has made it challenging to conclusively identify what the slugs are feeding upon. This challenge is further confounded due to lack of field observations for some genera, particularly *Plakobranchus*. Most likely due to its preferential use of diminutive taxa, *Plakobranchus* is almost never observed on siphonous green algae; they are almost always found on or burrowed in sand (personal observations). While it is unlikely that kleptoplast diversity and food species are not directly correlated, it is possible that the mechanics that facilitate kleptoplastidy are sophisticated

enough to preferentially select ideal chloroplasts for long-term retention. This is another particularly interesting avenue for future research: what are the mechanics that allow sacoglossans to identify and sequester chloroplasts instead of digesting them with the rest of the cellular contents? While still contentious (Pierce et al., 2011; Schwartz et al., 2014), it has been fairly well documented that it is not a result of horizontal gene transfer between algal source and slug host (Bhattacharya et al., 2013; Wägele et al., 2011), but other possibilities are yet to be explored. Lastly, now having a catalog of the diversity of algae utilized as kleptoplast sources and the limits of the host selection limitations of *Plakobranchus* cf. *ianthobapsus*, what are the drivers of these limitations? While the obvious hypothesis is anatomical limitations of their teeth and radula in relation to their preferential sequestration from diminutive species, this limitation is moot when considering that juveniles of all siphonous green algae are likely uniaxial, diminutive "green fingers." Thus, there are probably additional drivers that have yet to be defined.

7.1.2 Algal systematics, species delimitation, & invasive species detection

There are three clear limitations to the *Avrainvillea* systematics presented here: 1) only seven of the 37 currently recognized species comprising the genus are represented by type specimens in the molecular phylogenetic analysis, 2) there is poor coverage of all currently recognized species in general due to limited published data for the genus and costly nature of making representative collections from around the world, and 3) the multi-locus framework does not incorporate regions from outside the plastome. Given the invasive potential illustrated by at least three *Avrainvillea* species examined in this dissertation, there is a real need to address these shortfalls to produce a reliable framework for fast and accurate invasive species identification, to provide a robust phylogeny with global representatives to assess

possible source populations of introduced species, and to define phylogenetic species limits to make up for the challenges of morphological identification in this group.

As has been demonstrated in other studies (e.g. Jerde et al., 2011; Takahara et al., 2013; Xia et al., 2018), metabarcoding of environmental DNA (eDNA) is a powerful tool to detect the continued spread of *Avrainvillea lacerata* in the Main Hawaiian Islands. Continued efforts should be made to regularly sample epilithic communities in the high risk areas identified by Veazey et al. (In Revision), as well as urbanized estuaries and harbors (Wade et al., 2018) that are likely susceptible environments and points-of-entry for non-native species. Lastly, there should be continued engagement with the community, stakeholders, and representatives of state agencies to continue identifying and monitoring organisms as they enter Hawai‘i’s marine environments.

7.2 Final Remarks

Exploration of plant-animal interactions is an important avenue to explore a diverse array of questions, from building diversity inventories, to herbivore ecology and evolution, to invasive species detection and management. Perhaps the most elegant component of this research is the marrying of classic and modern approaches. Without the use of traditional feeding studies and bioassays, the support for preferential sequestration of chloroplasts from cryptic, diminutive algal species by *Plakobranchus* cf. *ianthobapsus* would remain only a suggestion from the results produced by molecular assessment of kleptoplasts . And when these molecular techniques fall short in their ability to fully capture diversity, the advent of high throughput sequencing of DNA amplicons provides an affordable, thorough exploration of epilithic algal communities.

The same can be said in terms of algal systematics – classic approaches using the assessment of type specimens to confidently identify species, particularly those that have

spread beyond their native ranges, are essential to provide reliable taxonomic information. But when morphology alone is unreliable, and the descriptions of many of these species were made nearly 150 years ago, it is essential to utilize modern genomic approaches that are capable of sequencing the entire target genomes with the available degraded DNA. Particularly with morphologically plastic taxa like siphonous green algae, the incorporation of both type material and next generation sequencing techniques allows the most thorough systematic assessment possible, and therefore provides the most confident identifications, particularly of invasive species.

7.3 References

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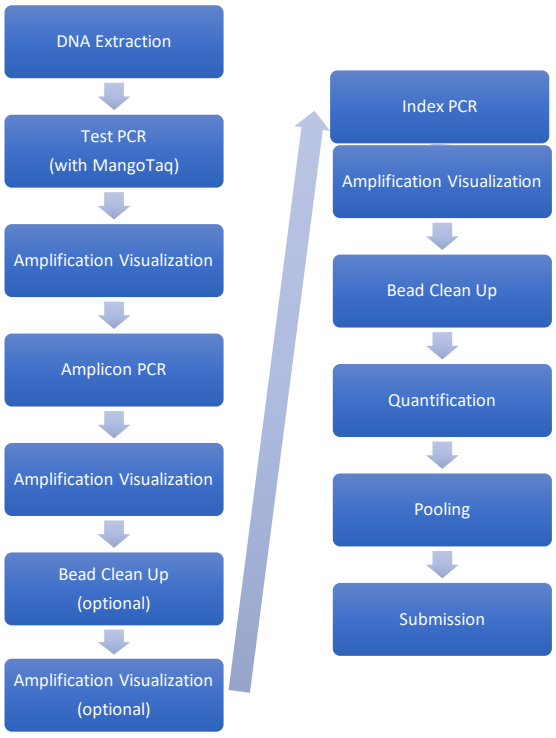
APPENDICES

Appendix 1. Library preparation protocol with amplicon and barcode sequence design.

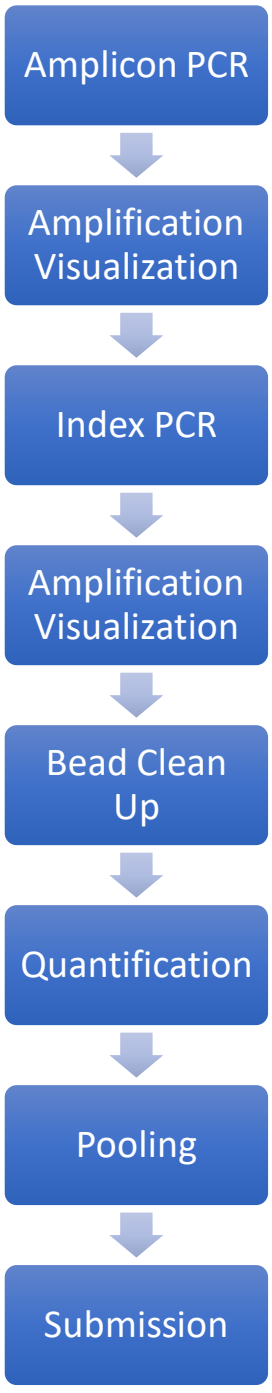
Next-Generation Sequencing *rbc*L Library Preparation for Illumina MiSeq System

(adapted from "16S Metagenomic Sequencing Library Preparation: preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system" protocol)



Alternatively, if you are familiar with your samples (i.e. confirmed amplification success, little to no dimer in PCR, etc.) you can use the following scheme, which was utilized for the *rbc*L kleptoplast library preparation.

IMPORTANT



Keep in mind that you will want to have an extraction negative sample (go through all of the extraction steps but without DNA) and have a PCR negative that will run through both the amplicon and index PCRs and independent clean-up steps. This

means each submission should have at least two more samples in addition to your experimental samples. Later, any data from these should be removed bioinformatically from your final dataset.

List of Supplies (+ Ordering Information)

Adapted amplicon primers (gene-specific primers with Illumina adapter overhang nucleotide sequences added)

Forward overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence] = TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AAAGCNGGKGTWAAAGAYTA

Reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence] = GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCAWCGCATARAWGGTTGHGA

Genomic DNA (eluted with ultrapure water) – make a 5-10 µl aliquot of your extracted genomic DNA to work with during your library preparation; numerous freeze-thaws will potentially degrade your DNA, especially since it is in water and not a buffer.

KAPA HotStart PCR Kit, with dNTPs (Roche Cat. No 07958897001, 250U, $263.00) or another high-fidelity Taq (i.e. Q5 High-Fidelity 2X Master Mix or Life Technologies Platinum DNA polymerase)

96-well PCR plates

Magnetic beads for PCR clean-up – GE Healthcare Sera-Mag Magnetic Speedbeads Carboxylate-Modified (Fisher Scientific, cat. # 09-981-123 [15 ml]) further processed by Amend lab (see Appendix, MagNA Protocol)

Freshly prepared **80% ethanol**

Magnetic bead separation block (V&P Scientific, Inc., cat. # VP 771HH-R, R version Flick & Blot for all standard 96-well microplates)

Blotting Paper & Absorbent Pad for hand-held blocks (V&P Scientific, Inc., lint-free blotting paper, cat. # VP 522B/pack of 10 or VP 522B-100/pack of 100, absorbent polypropylene pad, cat. # VP 540DB2/pack of 10 or VP 540DB2-100/pack of 100)

PCR water

Nextera XT Index kit (Illumina , Nextera XT Index kit, cat. # FC-131-1001 [24 indexes, 96 samples] or FC-131-1002 [96 indexes, 384 samples]) OR **non-proprietary i5 and i7 primers** (see Appendix, Dual Indexing, i5 and i7 Index Codes)

Qubit dsDNA assay kit, BR (broad range, quantitation range of 2-1,000 ng, quantitation of genomic and miniprep DNA samples) or HS (high sensitivity,

quantitation range of 0.2-100 ng, quantitation of PCR products, viral DNA, or samples for NGS), (Life Technologies, BR assay kits: cat. # Q32850 [100 assays] or Q32853 [500 assays], HS assay kits: cat. # Q32851 [100 assays] or Q32854 [500 assays])

Thin-walled, clear 0.5-ml PCR tubes for Qubit assay (VWR, Axygen PCR-05-C tubes, cat. # 10011-830 or Life Technologies, Qubit assay tubes, cat. # Q32856)

AMPLICON PCR

This step uses PCR to amplify template of a DNA sample using region of interest-specific primers with overhang adapters attached. IMPORTANT: this MasterMix is a guideline using KAPA HotStart PCR Kit only, follow the instructions particular to the polymerase you are using for a 25 µL reaction). Be sure to include your extraction and amplification controls, as well as a positive control, if possible.

Proceed as a regular PCR set-up but simply using the following reagents:

Reagent	**1X**
PCR Water	16.0 µl
Fidelity Buffer (5X)	5.0 µl
Adapted forward primer (10 µM)	0.75 µl
Amplicon Reverse primer (10 µM)	0.75 µl
dNTPs	1.0 µl
HiFi HotStart DNA Polymerase	0.5 µl
Genomic DNA (in water)	1.0 µl
Total	25 µl

Thermoocycler program for the *rbc*L region (Wade & Sherwood 2017):

95°C for 2 min

25 cycles of:

98°C for 20 s

47°C* for 15 s

72°C for 30 s

72°C for 5 min

4°C for ∞

Visualize PCR products (4.0 µl PCR product + 1 µl loading dye) in a 1.5% TBE-agarose gel with DNA ladder to see if successful (check general size range of bands, etc.), may or may not see a band at this stage – these products will be used to seed the Index PCR.

PCR CLEAN-UP USING MAGNETIC BEADS (optional)

This step uses magnetic beads to purify the amplicon away from free primers and primer dimer species (see Appendix, MagNA Bead Prep Protocol for more information). Prior to beginning the clean-up of your amplicon PCR products, it is recommended that you determine the DNA:bead ratio best for your samples and region. You can do this using a sample from your test PCR that amplified well, and testing at least .8x, .7x, .6x, and .5x to see which has the cleanest, yet still bright

band. For the *rbc*L amplicon, a 1:0.7 µl ratio was used. For example, for 20.0 µl of PCR product, add 14.0 µl of beads.
Bring magnetic beads to room temperature (they will need to be stored in the refrigerator).

Make sure all amplicon PCR products are in a 96-well PCR plate.

Vortex beads for **30 seconds** to make sure that beads are evenly dispersed before adding the beads to your products.

Gently pipet up and down **10X**.

Repeat steps 3 and 4 for each sample.

Incubate at room temperature for **5 minutes**.

Place the plate on magnetic block and wait for **2 minutes** or until supernatant has cleared.

Using the Flick & Blot design of this specialized magnetic bead separation block, simply flick out the liquid over the appropriated waste container for minimal bead loss during liquid removal step.

Appendix 2. QIIME pipeline commands used for processing of single, unmerged reads.

##
###############

#Commands for processing illumina amplicon individual, unpaired reads with Galaxy and QIIME

##
###############

Useful commands:

What is installed Macqiime? print_qiime_config.py -t

#Combine/merge fasta files into one master file (for QC)
cat *.fastq > merged.fastq

#Edit file names within file
sed 's/old file name/new file name/' <old file.fasta >new file.fasta

#Cpunt something in a file, e.g. # of seqs
grep -c ">" <fasta file with chimeras>

#To open QIIME, open a terminal window and type "macqiime"

#QC & trimming using Galaxy
Upload individual sequence files to Galaxy using CyberDuck; cannot use merged files if you want to be able to separate data by sample subsequently.

Trim using Trimmomatic using the following settings (order matters!):
HEAD CROP: 20 (to remove amplicon primer)
SLIDING WINDOW: 4, Q 33 (Note: less stringent Q 20 used for controls to ensure complete removal of contaminant seqs)
MINLEN: 205 (75% of 300, -20bp of amplicon primer)

Export trimmed sequence files and rename each file to reflect fasta (not fastasanger)

Find/Replace "fastqsanger" with "fastq" file type

#Demultiplex seqs with multiple_split_libraries_fastq.py
multiple_split_libraries_fastq.py -i <directory to file with all of the trimmed seqs> -o <directory to split_libraries folder> -m sampleid_by_file

Note: output file format from Galaxy works when trimmed to just “fastq”
Note: If any samples returned empty after QC, it will cause an error in this step

#Database Fasta File Clean-Up
clean_fasta.py -f <database> -o <output directory>

Database cannot have any "-" as gaps

#Chimera Check
identify_chimeric_seqs.py -i <fasta file of demultiplexed data> -m usearch61 -o <output directory> -r <reference database fasta file>
#can use smaller subset database

#Filtering out chimeras
filter_fasta.py -f seqs.fna -o seqs_chimeras_filtered.fna -s usearch_checked_chimeras/non-chimeras.txt
#output needs to be a file, not a directory

#pick OTUs with uclust
pick_otus.py -m uclust -i <seqs_chimeras_filtered.fna-o ph> -0 <directory> -s 0.95

#Picking representative sequences
pick_rep_set.py -i <input txt file of OTUs, output from pick_otus.py> -f <input file of seqs, fasta file with chimeras removed or non-match.fna> -m <rep set picking method, most_abundant> -o <output file.fna>

#output needs to be a file, not a directory
#search for the file name (since it will be placed elsewhere) and move it to an appropriate, findable folder, if an appropriate directory was not initially selected

#Assign Taxonomy
assign_taxonomy.py -i <rep set sequences> -t <file mapping names to seqs for ref set> -r <reference sequence dataset> -m <assignment method, blast> -o <output directory>

#make OTU table
make_otu_table.py -i <output from pick_otus.py> -o <output file with extension .biom> -t <txt file output from assign_taxonomy.py>

#filter singletons from OTU table
#filter_otus_from_otu_table.py -i <input file> -o <output file, .biom> -n <min # of samples, 2>

Create tab-delineated version of above OTU table
biom convert -i table.biom -o table.from_biom.txt --to-tsv --table-type="OTU table" --header-key="taxonomy"

#If you get the error "too many to unpack," try deleting the columns to the right of your actual info in the text file.

#Remove Negative Control OTUs

#1. Copy and paste Negative and PCR control columns from tab-delimited, filtered OTU table.

#2. If there are several different combinations of negative extraction and pcr control, make individual text files for each using Excel.

#3. Parce out your samples into blocks based on their controls. Save a text file with just the sample names.

#4. Filter samples from master OTU table
filter_samples_from_otu_table.py -i <otu table from "filter singletons…" step above.biom> -o sample_block_name.biom —-sample_id_fp <sample_names.txt from #4>

#5. Filter Negative control OTUs from sample block OTU table (#5)
filter_otus_from_otu_table.py -i <sample_block.biom (#5)> -o <sample_name_clean.biom> -e negative_control

#6. Confirm that proper samples were used and extracted
biom summarize-table -i <sample_name_clean.biom>

#7. Merge all final OTU tables.
merge_otu_tables.py -i otu_table1.biom,otu_table2.biom… -o merged_otu_table.biom

#8. Create text file of remaining OTU names.

#9. Filter final OTUs & sequences for downstream phylogenetics.
filter_fasta.py -f <.fna w/o chimeras> -s <txt file from #8>

BETADIVERSITY ANALYSES

#1. Create a folder for "Betadiversity" & "Betadiversity_files"

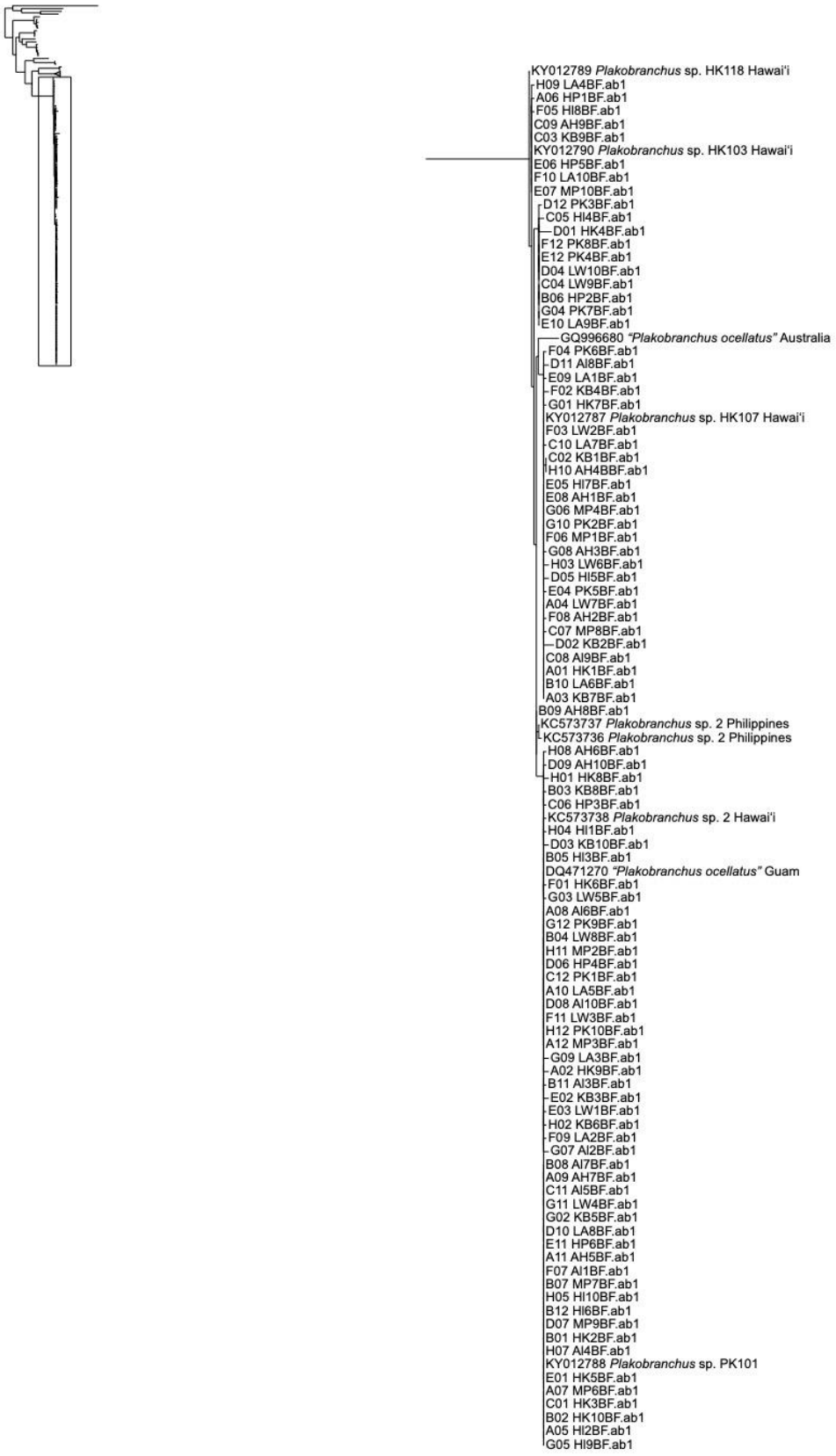
#2. For "Betadiversity files" you will need:
- UPGMA tree
- BIOM OTU table (#7 from "Remove Negative Control OTUS")
- Output director (e.g. Betadiversity folder)
- Mapping file with metadata
- pre-determined rarefaction number (what is the lowest number of sequences included in a sample?)

#3. Run a UPGMA tree in Geneious and export as a Newick tree.

#4. Conduct jack-knifed beta diversity analyses (jack-knifing provides confidence limit elipsoids)
jackknifed_beta_diversity.py -i <.biom file> -o <betadiversity folder directory> -m <mapping file> -t <UPGMA.newick> -e <rarefaction #> -f

#5. Insert a biplot over the beta diversity PCoA
make_emperor.py -i <principal coordinates file from unweighted Unifrac folder from jackknifed_beta_diversity.py> -t OTU_table.txt -m mapping_file.txt -o biplot -n # of taxa to display in biplot

Appendix 3. Maximum Likelihood-estimated phylogeny of the coxI barcode forward reads only (MG273316-410) of *Plakobranchus* cf. *ianthobapsus* populations in the Main Hawaiian Islands populations sampled in summer 2015. The largest intraspecific distance within the *Plakobranchus* cf. *ianthobapsus* clade produced by this analysis was 3.73% (~21 bp). Scale bar = number of substitutions per site.



Tree scale: 0.01

Appendix 4. *Avrainvillea* sp. records and site information for the 2014 seagrass survey in Honolulu Harbor.

Dive site	**Latitude**	**Longitude**	**Dredge or Control**	**Water Depth (m)**	***Avrainvillea* present**
1	21°18.0423'N	-157°52.14738'W	Dredge	10.4	
2	21°18.02412'N	-157°52.14306'W	Control	12.5	
3	21°18.03018'N	-157°52.13796'W	Dredge	12.5	
4	21°17.99988'N	-157°52.13736'W	Control	14.6	
5	21°17.96316'N	-157°52.18926'W	Dredge	11	
6	21°17.93676'N	-157°52.1799'W	Control	12.2	Yes
7	21°18.12077'N	-157°52.14564'W	Dredge	9.8	
8	21°18.11826'N	-157°52.13094'W	Control	13.1	
9	21°17.67558'N	-157°52.25802'W	Dredge	11.9	
10	21°17.6898'N	-157°52.2759'W	Control	16.5	
11	21°17'.74512'N	-157°52.31226'W	Dredge	14.3	
12	21°17.75214'N	-157°52.29264'W	Control	15.5	Yes
13	21°17.85102'N	-157°52.24866'W	Dredge	14.3	Yes
14	21°17.85264'N	-157°52.22544'W	Control	15.2	Yes
15	21°18.24468'N	-157°51.9408'W	Dredge	12.5	Yes
16	21°18.24108'N	-157°51.95244'W	Control	13.4	Yes

Appendix 5. *Avrainvillea* specimen collection and sequence information.

		Field/Herbarium Code								GenBank Accessions	
	ARS #	BISH #	BM #	TS #	GWS #	Collection Location	Collection Date	Collector	Determiner	*rbc*L	*tuf*A
Avrainvillea sp.	8291					Mediterranean Sea, Libya	7-Apr-12	A. El Fituri	R. Wade	MF872090	MF872115
Avrainvillea sp.	8292					Mediterranean Sea, Libya	7-Apr-12	A. El Fituri	R. Wade	MF872091	MF872116
"Avrainvillea amadelpha"	8383					Natatorium Reef, Waikiki, O‘ahu	21-Dec-12	G. Kraft	R. Wade	MF872087	MF872112
"Avrainvillea amadelpha"	8418					Hunakai Beach; east of pipe, <1 m	23-Mar-13	R. Wade	R. Wade	MF872096	MF872124
Avrainvillea sp.	8484					Great Chagos Bank, Indian Ocean; 15m, Fringing Reef, 6°10.346' S, 71°19.988 E	7-Mar-13	D. Wagner	R. Wade	MF872092	MF872117
Avrainvillea erecta	8485					Bunaken Island, Sulawesi; Intertidal, Sand Flats	Mar-12	H. Spalding	R. Wade	MF872094	MF872119
"Avrainvillea amadelpha"	8510					Barber's Pt., Oahu; 120 feet (36 m), thin layer of sediment on flat carbonate.	23-Jul-13	M. Ross	R. Wade	MF872089	MF872114

"Avrainvillea amadelpha"	8511					Barber's Pt., Oahu; 120 feet (36 m), thin layer of sediment on flat carbonate.	23-Jul-13	M. Ross	R. Wade	MF872093	MF872118
Avrainvillea erecta	8603					Eor, Murilo Atoll, Federated States of Micronesia, Chuuk State; LAT 8.685683333, LON 152.334283	2-Aug-08		R. Wade	MF872076	MF872101
Avrainvillea riukiuensis	8608					Moch Island, Satawan Atoll, Federated States of Micronesia, Chuuk State; LAT 5.479666667, LON 153.539667	17-Apr-08		R. Wade	MF872078	MF872103
Avrainvillea erecta	8609					Satawan Atoll, Federated States of Micronesia, Chuuk State; LAT 5.374, LON 153.5561	16-Aug-08		R. Wade	MF872088	MF872113
Avrainvillea erecta	9414	7682				Ke‘ehi Lagoon, O‘ahu; 25-40 m	22-Apr-17	M. Ross	R. Wade	MF872083	MF872108
Avrainvillea erecta	9417	7682				Ke‘ehi Lagoon, O‘ahu; 25-40 m	22-Apr-17	M. Ross	R. Wade	MF872080	MF872105
Avrainvillea erecta	9418	2682				Ke‘ehi Lagoon, O‘ahu; 25-40 m	22-Apr-17	M. Ross	R. Wade	MF872081	MF872106
Avrainvillea erecta	9429	7682				Ke‘ehi Lagoon, O‘ahu; 25-40 m	22-Apr-17	M. Ross	R. Wade	MF872084	MF872109
Avrainvillea erecta	9431	7682				Ke‘ehi Lagoon, O‘ahu; 25-40 m	22-Apr-17	M. Ross	R. Wade	MF872082	MF872107
Avrainvillea erecta	9432	7682				Ke‘ehi Lagoon, O‘ahu; 25-40 m	22-Apr-17	M. Ross	R. Wade	MF872085	MF872110

Avrainvillea erecta	9436	768338				Entrance channel to Honolulu Harbor, Honolulu, O‘ahu	15-Oct-14	K. Peyton	R. Wade	MF969093	MF969095
Avrainvillea erecta	9437	768339				Entrance channel to Honolulu Harbor, Honolulu, O‘ahu	15-Oct-14	K. Peyton	R. Wade	MF969094	MF969096
Cladocephalus sp.	8897			1796		Mote Marine Laboratory Lagoon/Summerland Key, Fronting Lagoon, <2 m	11-13-Sept-13	T. Sauvage	R. Wade	MF872072	MF872097
Cladocephalus sp.	8899			1820		Venture Key, across Mote Lab, <2 m	12-Sep-13	T. Sauvage	R. Wade	MF872073	MF872098
Avrainvillea sp.	8894			1356		Haemida: Iriomote SIte 3, 1-2 m	24-Jul-12	T. Sauvage	R. Wade	MF872074	MF872099
Avrainvillea sp.	8895			1543		Sesoko Island, Okinawa summer site 4, 1-2 m	4-Aug-12	T. Sauvage	R. Wade	MF872075	MF872100
Avrainvillea erecta	8886			1256		Hamahiga resort beach: Okinawa site 2 Summer	24-Jun-12	T. Sauvage	R. Wade	MF872077	MF872102
Avrainvillea sp.	8885			787		Site 15 Suez artificial rocky pier, 1 m	11-Jul-11	T. Sauvage	R. Wade	MF872079	MF872104
Avrainvillea sp.	8884			786		Site 15 Suez artificial rocky pier, 1 m	11-Jul-2011	T. Sauvage	R. Wade	MF872086	MF872111

Avrainvillea sp.	8891			1331		Sonai, Iriomote Site 1, Okinawa	24-Jul-12	T. Sauvage	R. Wade	MF872095	MF872123
Avrainvillea calathina					22850	Malabar Reef, Lord Howe, AU	21-Nov-10		R. Wade		MF872120
Avrainvillea calathina					22886	Malabar Reef, Lord Howe, AU	21-Nov-10		R. Wade		MF872121
Avrainvillea calathina					23474	Algae Hole North, Lord Howe, AU	21-Nov-10		R. Wade		MF872122
"*Chloroplegma papuanum*" - *A. erecta* heterotypic synonym			561613			Papua, Indonesia	May-1872	O. Beccari	Zanardini	MH938452	

Appendix 6. Morphological characters used to identify the new *Avrainvillea* specimens and comparison with related species. Reference species characters retrieved from the descriptions provided by Olsen-Stojkovich (1985). Bolded character text represent character congruence with the newly recovered species from Hawai‘i.

			***Avrainvillea* sp. (this study)**	***A. asarifolia***	***A. clavitiramea***	***A. elliotti***	***A. erecta***	***A. gardineri***	***A. nigricans***	***A. obscura***	***A. pacifica***
Macroscopic	**Plant**	**Habit**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**	**Psammophytic; primarily solitary individuals with differentiated holdfast, stipe, blade**
	Blade	**Width (cm)**	**1.80-(5.60)-9.50**	**5-6**	Up to 6		2-6	12-20	2-25	3-6	5-8
		Length (cm)	**2.10-(8.03)-11.50**								
		Shape	**Sub-reniform to -cuneate**	Orbicular to reniform			**Reniform to subcuneate**	Rhomboid to orbicular	Cuneate, obvoate, or rhomboid	**Cuneate, reniform, or rotunda**	**Reniform, occasionally cuneate**

										te; unbranched	
		Morphology	**Thin; lightly zonate; olive green to fulvous (when dried); unbranched**	Thick, velutinous; olive; unbranched, can be branched at the base	Spongy, subvelutinous; olive brown; never zonate; unbranched	Spongy; brownish-olive; unbranched, split to form lobes; zonate	**Thin to spongy; olive to yellowish; unbranched**	**Thin, velutinous, translucent; olive; unbranched; zonate**	Thick, spongy, velutinous; brown to black; unbranched or branched once; never zonate	**Spongy to subvelutinous; seldom zonate; olive to dark green**	**Spongy, velutinous; olive green; unbranched; zonate**
		Margin	**Sometimes smooth, but loose siphons; sometimes lacerate**	**Smooth**	**Smooth to slightly lacerate**	Smooth or lobed	**Smooth, rarely lacerate**	**Lacerate**	**Smooth or slightly eroded**	**Smooth to slightly irregular**	**Smooth**
	Stipe	**Width (cm)**	**1.00-(1.37)-1.80**								
		Length (cm)	**0.50-(1.37)-2.50**	Up to 12	Up to 6	**2-5**	**1-2**	**1-2**	**16**	**0.30-0.80**	**1-2**
	Frond (Bla	**Width (cm)**	**3.60-(6.97)-10.80**								

	de + Stipe)	**Length (cm)**	**3.90-(9.83)-14.00**	18-22	**2-3**	**6-10**	**Up to 6**	**Up to 30**	**3-20**	**3-8**	**4-8**
	Hold fast	**Width (cm)**	**1.60-(2.67)-3.80**								
		Length (cm)	**3.10-(3.83)-5.00**					**Up to 7**		**4-20**	
		Morphology	**Submerged, rhizomatous**	**Rhizomatous, submerged**	**Rhizomatous, submerged**	**Rhizomatous, submerged**	**Rhizomatous, submerged**	**Rhizomatous, submerged**	**Rhizomatous, submerged**	**Rhizomatous, submerged**	**Rhizomatous, submerged**
Microscopic	**Siphons (Assessed at growing margin, mid-blade, stipe, and holdfast)**	**Color**	**Translucent, olive green, or fulvous**	**Olive**	**Olive brown**	**Olive**	**Bright yellow, orange-brown**	**Olive**	Dark brown to black	**Olive to light brown**	**Olive or hyaline**
		Morphology	**Primarily cylindrical to slightly torulose; pseudocortex absent**	**Cylindrical to torulose; pseudocortex absent**	**Cylindrical, irregularly torulose**	**Irregularly cylindrical or torulose; pseudocortex absent**	**Cylindrical**	**Cylindrical or torulose; pseudocortex present**	Predominantly moniliform, sometimes cylindrical or torulose	**Cylindrical, intermittently torulose**	Torulose; pseudocortex absent
		Width (µm)	**11.1-(25.4-59.1)-93.1**	**(20-30)**	**19-(38)-57**	**15-(20)-38**	**(28-47)-66**	**(19-28)**	**19-(38-47)-66**	**28-(38-47)-56**	**(19-28)**

		Supradichotomy width (µm)	**5.7-(11.7-22.7)-30.0**								
		Supradichomy morphology	**Deeply & acutely constricted**	**Deeply constricted**	**Deeply constricted**	**Deeply constricted with subsequent swelling and secondary constriction**	**Deeply constricted**	Shallowly constricted	**Deeply constricted**	**Deeply & acutely constricted**	Shallowly constricted
		Apex shape	**Primarily rounded**	Tapered, thread-like	Clavate to subclavate	**Rounded, sometimes clavate**	**Rounded**	**Rounded**	**Rounded**	**Rounded or clavate**	Twisted & hooked
		Apex width (µm)	**13.1-(27.7-57.2)-69.4**		(57)-100	(15-20)		(12-15)		(56)-125	